FLUORCHEM Q
USER MANUAL

Alpha Innotech

Gel Imaging Systems for Life Sciences

Introducing AlphaView®

AlphaView provides the utmost ease of use while offering comprehensive and versatile tools for capturing, analyzing, and annotating images. With a simple to use graphical user interface coupled with new and improved features, Alpha Innotech has pioneered the most intuitive image capture and analysis software available.

AlphaView's new and improved features include multiple image viewing, the ability to save analyses, and an enhanced movie mode. With our suite of analysis tools, you can perform molecular weight calculations, R_f determination, lane profile densitometry, multiplex band analysis, microtiter plate reading, object distance measuring, gel scoring, and automatic colony counting.

In addition, AlphaView's image optimization tools can adjust contrast automatically or manually, convert images from positive to negative using digital filters, apply false color, and utilize many other techniques to clarify difficult-to-see portions of the image. Notes, labels, arrows, lines, and other drawing tools can be recorded directly onto the image using AlphaView's annotation features. Annotations are superimposed on the image upon hard copy printing and can either be saved as a template file or as part of the image itself. All AlphaView features are accessible via convenient on-screen buttons and menus in an intuitive interface.

Images can be printed using a 256-level gray scale thermal printer or any printer with a Windows® driver. The low-cost, high-quality prints are ideal for lab notebook records or publication

About This Manual

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- **`Courier font`** indicates an entry that is typed.
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- Bolded **NOTE:** indicates key points and useful hints
- Bolded **CAUTION:** indicates actions that may either harm the system or affect the data quality.
- Icons or buttons to be pushed are placed next to their respective descriptions in the text.

Questions or Comments?

For questions or comments, please use the following contact methods:

For general information:

- Email: info@alphainnotech.com
- Phone: (510) 483-9620

For technical support:

- Email: support@alphainnotech.com
- Phone: (800) 823-0404

Business hours are Monday through Friday, 7:00 AM to 4:00 PM PST.

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Introduction to the FluorChem Q Image Analysis System

Introduction

The **FluorChem Q** system is designed specifically for the acquisition of high quality images from multicolor Westerns. The **FluorChem Q** produces uniform and consistent fluorescence images with the sensitivity and dynamic range needed for accurate quantification of multicolor Westerns. Together with the advantages of multicolor fluorescence labeling for Western blots, the **FluorChem Q** provides the most cost effective solution for your imaging requirements. The **FluorChem Q** includes all the features of the **FluorChem HD2** plus the light sources and emission filters preinstalled for multicolor fluorescence imaging. The **FluorChem Q** now provides outstanding performance in each of the most commonly used imaging modes for gels and blots: chemiluminescence, colorimetric, UV trans-fluorescence, and multicolor epi- fluorescence.

The FluorChem Q imaging systems are designed to meet the requirements of the most demanding chemiluminescence assays and also provide the most versatile fluorescence imaging system available today. **FluorChem Q** systems integrate the latest developments in camera and lens technology for imaging chemiluminescence and fluorescence samples with wide dynamic range and fast image acquisition speeds. The system is controlled by easy-to-use and intuitive software designed by Alpha Innotech. AlphaView™ software performs image analysis and archiving and can also prepare images for desktop publishing.

AlphaView™ software includes image optimization tools that adjust contrast automatically or manually. Converting the image from positive to negative, using digital filters, applying a false color map, or other such techniques can be used to clarify hard-to-see portions of the image. Notes, labels, arrows, lines, and other drawing tools can be recorded directly onto the image using the AlphaView™ software's annotation functions. Annotations are superimposed on the image upon hard copy printing and can either be saved as a template file or as part of the image. All AlphaView™ features are accessible via convenient on-screen buttons and menus in an intuitive, mouse-controlled interface.

AlphaView™ software also includes a broad array of analysis tools, including molecular weight calculation, R_f determination, lane profile, multiplex band analysis, quantitative PCR, microtiter plate reading, object distance measuring, gel scoring, and automatic colony counting.

FluorChem Q system is a complete package that includes all necessary hardware and software for image capture, enhancement, and analysis. AlphaView™ software does not require a dedicated computer system and can operate simultaneously with other office-related software, such as word processing programs, spreadsheets, and desktop publishing software.

Images can be printed using a 256-level gray scale thermal printer or any printer with a Windows® driver. The low-cost, high-quality prints are ideal for lab notebook records or for publication. A list of journals featuring published prints generated from AlphaView™ software is available from Alpha Innotech.

Chemiluminescent Detection

The **FluorChem Q** has the same outstanding capabilities for chemiluminescence detection as the **FluorChem HD2**. The **FluorChem Q** imaging system incorporates the same CCD camera, f/0.95 fast lens, and dark enclosure as the **FluorChem HD2** supporting quantitative chemiluminescent detection. Chemiluminescence is a well established detection technique for quantification of protein abundance on Western Blots. Chemiluminescence exploits the catalytic reaction of an enzyme and a peroxide-based substrate to produce a light signal with very low background as no illumination is required. The enzyme (e.g. horseradish peroxidase) is conjugated to a secondary antibody that binds to the primary antibody specific to the protein of interest. Chemiluminescence is widely used by researchers because it is much less hazardous than radioactivity while achieving equal performance.

Fluorescence Detection

The quantitative power of Western Blot analysis can be further improved by using fluorescence detection methods for many experiments. Single color fluorescence detection methods compare favorably to chemiluminescence in terms of cost and ease of use. While fluorescence may often have a higher level of background signal, fluorescence has an intrinsically linear response to protein level whereas the enzymatic reaction producing the chemiluminescence signal may locally saturate causing a “brownout”.

Multicolor fluorescence detection methods offer significant additional advantages for quantifying multiple proteins on a single blot. Multiple proteins can be accurately quantified using chemiluminescence or single color fluorescence detection methods only when the proteins are very similar in abundance and are also well resolved on the blot. There are many experimental situations where these conditions are not met.

With multicolor fluorescence detection methods up to three proteins can be accurately quantified using antibodies labeled with distinct fluorophores and imaged with the appropriate excitation and emission wavelengths. With multicolor fluorescence, you can resolve and quantify proteins that migrate in overlapping bands and also accurately quantify proteins with much different levels of relative abundance. In particular dim bands adjacent to bright bands can be resolved and quantified.

For example, the protein used as loading control is usually of much higher abundance than the protein of interest. Finding a single exposure time that detects both quantitatively may not be feasible when using a common detection method for each protein. By labeling the loading control and experimental proteins with different fluorophores, the labeling conditions and image acquisition settings can be adjusted appropriately to bring each of the proteins into the quantitative dynamic range of the image. The relative expression levels of each protein can then be analyzed in comparison to relevant positive controls and then compared to each other.

There are many labeling protocols available for multicolor fluorescence Western blots. Choosing the optimal labeling protocol for a specific application depends upon the nature of the primary antibodies used and other factors. One general multicolor protocol involves using primary antibodies raised in different species for each protein followed by the corresponding species specific secondary antibodies. For example, a generic combination of primary and secondary antibody combinations is shown in Figure 1. The antibodies chosen should be tested for cross-reactivity to other antigens, non-target proteins and other antibodies utilized in the assay.

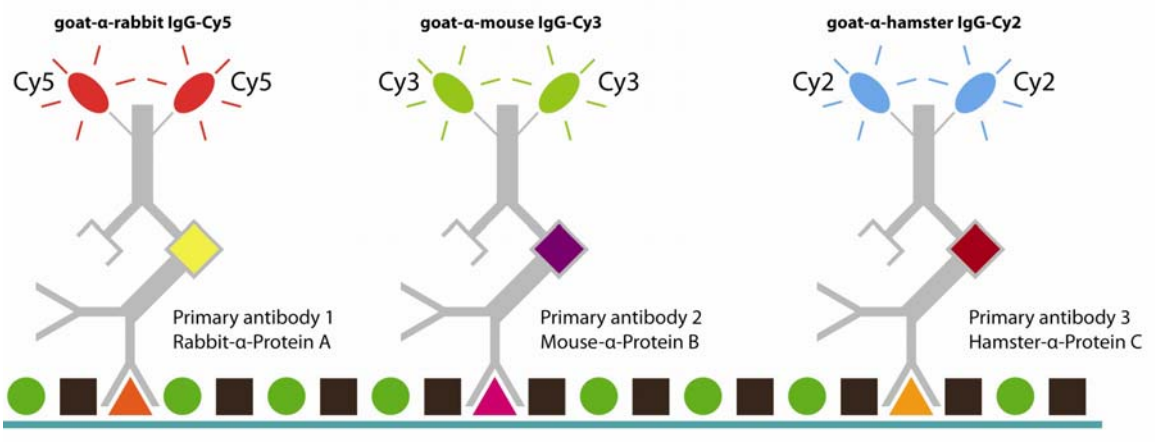

Figure 1: Typical multicolor fluorescence labeling anti body combination.

The advantage of using multicolor fluorescence labeling protocols is that the final signal levels for each protein can be balanced for accurate quantitative analysis of each labeled protein. For example, the concentrations of the secondary reagents can be adjusted while maintaining the optimal concentrations of primary antibody for each protein. This is especially useful when the proteins vary significantly in abundance. Often the loading controls are much more abundant that the experimental proteins.

It is also feasible to actually monitor the fluorescence or immunostaining procedures to determine the optimum processing time by placing the container with the blot immersed in the staining solution directly into the **FluorChem Q** and acquiring an image. After processing, dry blots often produce images with superior contrast to images obtained from wet blots for the CY dyes. Some fluorophores, such as FITC, fade significantly upon drying. Auto fluorescence levels may be high for some nitrocellulose or PVDF (polyvinyl fluoride) membranes, especially under blue light illumination. Immobilon FL (Millipore Corporation), Hybond LFP and Hybond ECL (GE Healthcare) are low auto fluorescence PVDF and nitrocellulose membranes suitable for visible fluorescence applications with auto fluorescence levels as low as NIR fluorescence.

Advantages of the FluorChem Q

The **FluorChem Q** includes preinstalled Blue, Green, and Red LEDs, Blue, Green, and Red excitation and emission filters, 50mm F/0.95 lens and HD2 camera, for acquisition of high quality single color and multicolor fluorescence images. The key feature of the **FluorChem Q** is the high power LED illumination system that produces a consistent illumination field and uniform fluorescent images.

A second key feature of the **FluorChem Q** is the direct acquisition of multichannel images. By defining an image acquisition protocol, all the system settings for sequential acquisition of multiple images of the sample are saved and executed automatically. There is no need to manually switch between LEDs or filter positions during acquisition. Bias, dark and flat field calibration and image registration are applied automatically during image acquisition for a rapid and streamlined image acquisition procedure.

Fluorescence images acquired by the **FluorChem Q** may be either single channel or multichannel. A single channel image contains a single exposure at one specific combination of excitation and emission settings and is used for blots labeled with a single fluorophore. Single channel images have a 16 bit depth. A multichannel image contains multiple exposures, each exposure at a different combination of excitation and emission settings. Multichannel images are

used primarily for samples that have been labeled with two or three fluorophores, such as CY2, CY3 and CY5. Multichannel images are acquired in a 48 bit RGB format with 16 bit depth in each channel.

The **FluorChem Q** includes a number of advanced image acquisition features designed for optimal image acquisition of multicolor fluorescence Western blots:

Three spectral detection channels optimized for the most commonly available fluorescence reagents (CY2, CY3 and CY5):

Channel	Excitation	Emission	Dye
Blue	475/42	537/35	CY2, Alexa 488, DyLight 488, FITC
Green	534/30	606/62	CY3, Alexa 546, DyLight 549
Red	632/22	699/62	CY5, Alexa 647, DyLight 649, also Red: Cy5.5, Alexa 680, DyLight 680

The acquisition of multicolor fluorescence images follows the same overall process as the acquisition of single channel images. The objective is to acquire an in-focus image of the blot with the features of interest exposed to levels suitable for accurate quantitative analysis. In practice the presence of background signal arising from the blot and sample will reduce the assay dynamic range. Generally using auto expose will produce an optimal image if the brightest feature in the image is a feature of interest

Mouse Functions

The FluorChem Q System comes packaged with a two-button mouse. The left button activates functions and makes selections when using the software. In some cases, the right mouse button can recall or reactivate the function that was most recently assigned to the left mouse button.

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- Bolded **WARNING:** indicates actions that can potentially be harmful to the operator.
- Icons or buttons to be pushed are placed next to their respective descriptions in the text.

Questions or Comments?

The Alpha Innotech Corporation staff is available to respond to any questions or comments about the software. For questions, new software feature ideas, or general feedback, use the following contact methods:

- Email: info@alphainnotech.com
- Fax: +1-510-483-3227
- Telephone: **800-823-0404** or **+1-510-483-9620,**

Telephone support is available between 7:00 AM and 4:00 PM Pacific Standard Time.

Starting AlphaView™ software

To start AlphaView™ software from Windows, double-click the AlphaView™ Software icon on the windows desktop.

Figure 1.1 AlphaView™ shortcut

FluorChem Q Imaging System Setup

System Components

FluorChem Q System includes the following:

- High-performance, high resolution, CCD camera
- Manually operated 50mm f/0.95 lens or other optional lens
- Computer with keyboard, mouse, and monitor (premium specifications optional)
- Windows operating system (pre-installed)
- AlphaView™ image processing and analysis software (pre-installed and calibrated with computer and hardware system)
- MultiImage III ®FCQ light cabinet with UV transilluminator and white light fold-down transilluminator and interference filter
- Fast lens (optional)
- Epi-illuminating UV lights (optional 254nm or 365nm)
- Printer (optional)
- ChromaLight (optional)

Upon receiving the system, it is critical to check the enclosed packing list to verify that all components are properly included.

System Placement

As with all electrical instruments, the FluorChem Q System should be located on a table or bench top that is dry and stable and away from water, solvents, or corrosive materials. In addition, the system should be placed away from interfering electrical signals and magnetic fields. If possible, a dedicated electrical outlet should be used to eliminate electrical interference from other laboratory instrumentation.

Cable Connections

The cable connectors and their respective mating ports are keyed or unique for each connection to eliminate potential wrong mating. The connections are illustrated and described in section 1.3.

WARNING: Make sure that the power is OFF and all power cords are disconnected while connecting the cables and setting up the system.

Hardware Installation

All software, peripheral drivers, and operating systems come factory-installed. All components must be mated only to their correct ports during system installation.

Power Strip/Surge Protector Setup

Turn the power strip/surge protector power switch off. Plug the power strip/surge protector into a wall outlet (preferably a dedicated circuit) and turn the power on.

CAUTION: Do not plug the DE-500/DE-500FC MultiImage III light cabinet into the same power strip as the FluorChem Q System. Using a separate circuit is highly recommended. The cabinet must be turned on after the operating system has been completely loaded for the software to function optimally.

Computer, Monitor, Mouse, and Keyboard Setup

The computer must have the monitor, mouse, and keyboard connected in the correct ports (see Figure 2). A standard three-prong power cable should be plugged into the back of the computer and the power strip.

Connect the monitor's video cable to the monitor port on the back of the computer. If after-market video cards were preinstalled into your computer system, connect the monitor's video cable to the video card connector. Connect the monitor's power cable to the power strip.

The mouse and keyboard connectors are color-coded and icon identified. Attach the mouse and keyboard cables to the connectors on the back of the computer by matching the colors. Some systems have USB mouse and keyboards.

The computer is now ready to be turned on. Turn the computer on by pushing the power button on the front of the unit.

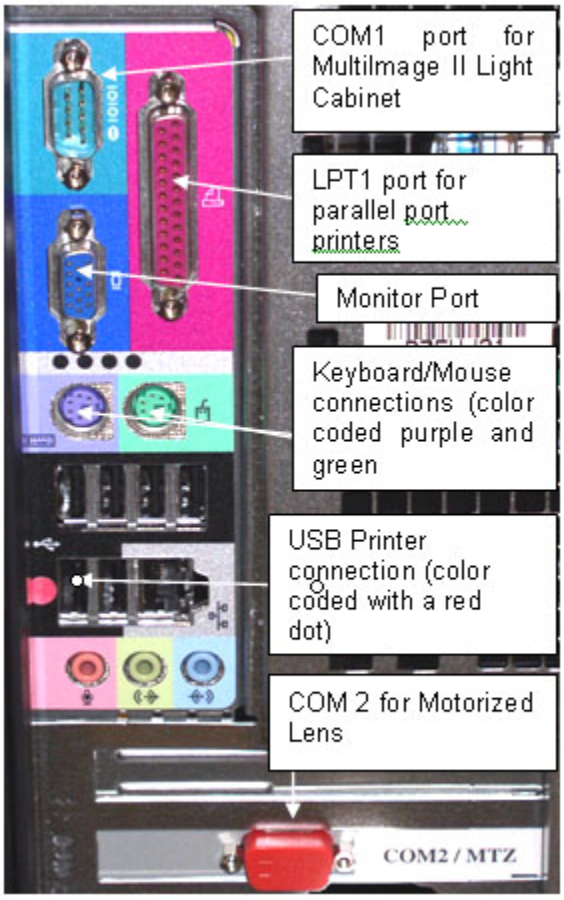


Figure 1.2 Computer, monitor, mouse and keyboard setup

Cabinet Installation Instructions

MultiImage™ III Light Cabinet (DE-500FCQ)

Figure 1.3 Pictured with the cabinet top (DE-500FCQ)

Cabinet Setup

When you remove the light cabinet from its shipping carton, it is already partially assembled. The camera mounting assembly is packed separately in the same container. The UV transilluminator and cabinet top are both packed in separate boxes. Make sure you have received all the hardware before discarding the shipping carton.

1. The largest box will be the DE500 cabinet. This box will include camera bracket, camera bracket gasket, RS-232 cable, magnetic pad and pen.

2. Set the entire cabinet assembly on a level, flat surface. (There are indentations to the bottom of the cabinet, please use them for lifting and placement of the unit. The cabinet weighs 80lbs with the UV transilluminator please take appropriate precautions in lifting and moving the light cabinet.) The footprint dimensions for the cabinet is 20" wide X 14" deep.

3. Open door and remove shipping foam from above and below fold down white light table. Note: camera bracket, gasket, RS-232 and magnetic pad/pen will be located in and around the shipping foam. Fold up white light table.
   Note: be sure to remove the inserts from the white sidelight bulbs.

4. Slide out UV tray. Unpack UV transilluminator and mount onto sliding tray (be sure to align UV transilluminator rubber feet into sliding tray open positions). Inside the cabinet, locate the power cord taped with RED tape. Please uncoil and insert into the power socket for the UV transilluminator

5. Plug the light cabinet into the surge protector, turn on the cabinet, and test each of the switches on the front of the cabinet to ensure that all connections were made properly. **Note: Do not plug a transilluminator or the MultiImage™ III light cabinet into the same power strip as the computer;** use a different circuit whenever possible**.**

6. FluorChem Q provides cabinet controls through software. An RS-232 cable that is supplied with the cabinet is required for this purpose. Please located the RS-232 cable insert it into the corresponding socket next to the power connect and power switch on the rear of the DE500 (see picture following page).

7. A 3 foot long 8 pin miniDIN cable connects the camera to the rear of the cabinet.

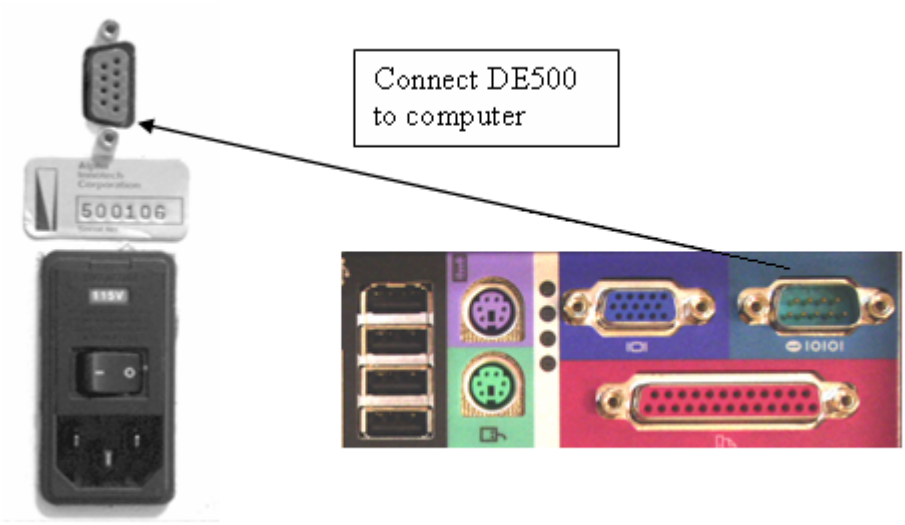


Figure 1.4 Cabinet Setup

The DE-500 and DE-500FC MultiImage Light cabinets connect to the back of the computer via an RS232 cable.

WARNING:
IF EQUIPMENT IS USED IN A MANNER NOT SPECIFIED BY THE MANUFACTURER, THE PROTECTION PROVIDED BY THE EQUIPMENT MAY BE IMPAIRED.

"CAUTION:
POWER SUPPLY CORD IS USED AS THE MAIN DISCONNECT DEVICE. ENSURE THAT THE SOCKET-OUTLET IS LOCATED/INSTALLED NEAR THE EQUIPMENT AND IS EASILY ACCESSIBLE"

Symbols:

Figure 1.5 Ultra Violet Light

WARNING: Please use protective equipment when using UV light. UV protective goggles, face shields, long sleeve lab coats can be obtained from major scientific distribution catalogs. (e.g. Fisher Scientific)

Figure 1.6 CAUTION: Risk of electric shock

Figure 1.7 HAZARD, please take appropriate precautions

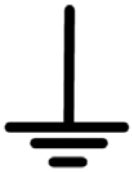
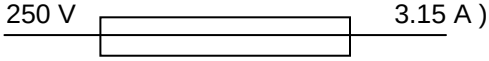


Figure 1.8 Earth (ground) Terminal



Fuse symbol

Power Ratings:

DE 500FCQ MultiImage™ III cabinet is rated at:

250V 50/60 Hz 3A

Fuse: 250V 3.15A

Camera Components and Installation

1) Connect the Camera cable, camera power supply and miniDIN connectors to the back side of the camera.

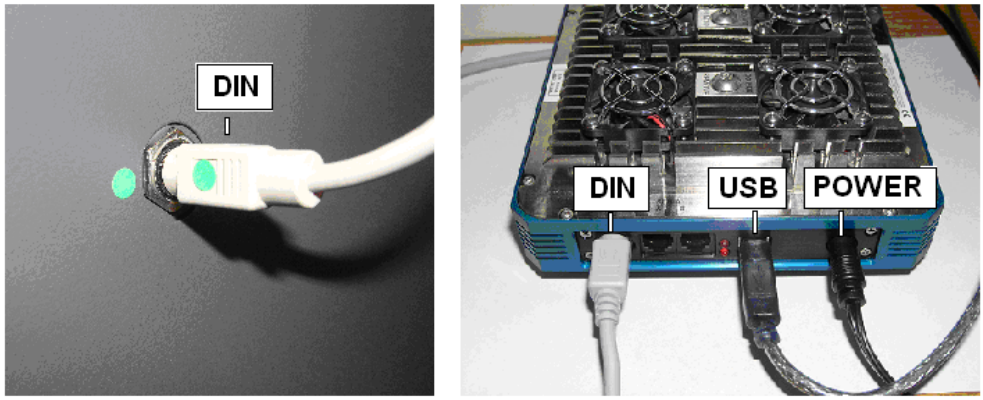


Figure 1.9 Camera Setup Step 1

2) Connect the power cable standard three-prong end to the power strip.

Figure 1.10 Camera Setup Step 2

3) Complete the installation by connecting the camera cable to the USB port.

Figure 1.11 Camera Setup Step 3

Connecting the Printer

The Mitsubishi P93DW (UB) printer connections are color coded for convenience (see figure in section 1.3.2). Plug the USB cable into the back of the printer and into the proper USB connector on the back of the computer. Plug in the standard three-prong power cable to the back of the printer and to the power strip, then turn the power on.

NOTE: The Mitsubishi P93D (UB) printer may be connected to any USB port on the computer; however, the driver may need to be manually re-installed depending on which USB port is used.

The Mitsubishi CP700 and CP770 printers connect via the on-board parallel (LPT1) port. Plug in the standard three-prong power cable on the back of the printer and into the power strip, then turn the power on. Set the printer up for color or black and white printing following the directions in the owners' manual supplied with the printer.

Power up Sequence

Once all connections are made between computer, cabinet, camera, printer and monitor power can be applied in the following sequence:

1) Monitor
2) Computer
3) DE500 cabinet
4) Camera power supply- the camera will begin to cool to –10C.
5) Printer

After the cabinet filter wheel has finished homing launch the AlphaView software to begin deep cooling of the camera (minimum cooling period is 30 minutes)

Starting AlphaView™ FC-Q System with AlphaView™ software

To launch the AlphaView™ Imaging System software from Windows, double-click the AlphaView™ System icon on the Windows desktop.

System Information

To display system information, select the About option in the Help menu. This button accesses a pop-up box.

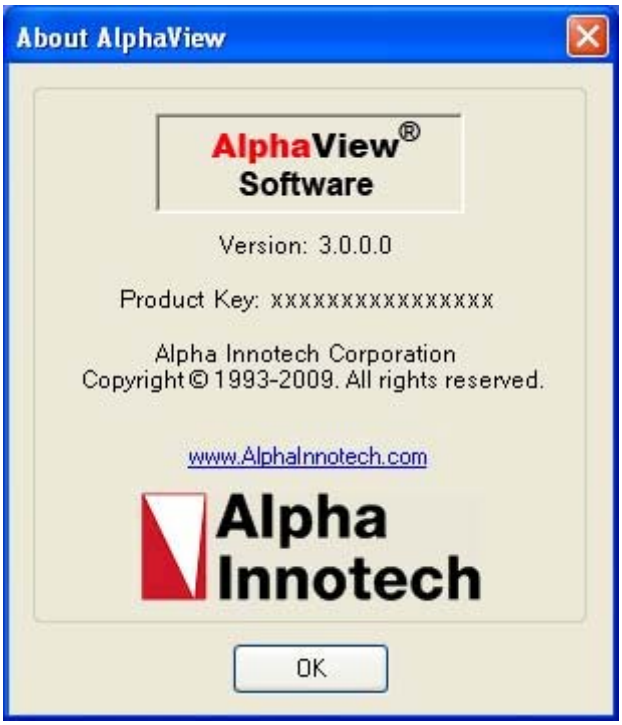


Figure 1.12 System Information

This box shows the Software version number. Use the information specific to your instrument and software when calling Alpha Innotech for technical support and software upgrades.

To close the box, click on the OK button.

Chapter 2. GETTING STARTED - BASIC IMAGING FUNCTIONS

When the FluorChem Q system computer is powered up, you can click on the FluorChem Q icon on the desktop to automatically open the AlphaView™ software. The following screen appears:

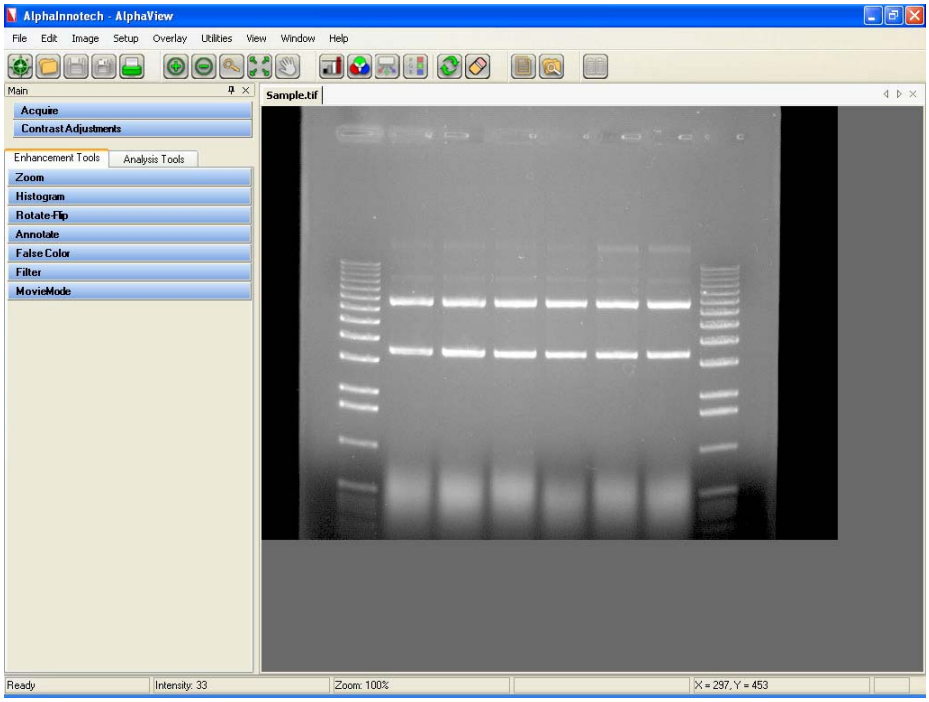


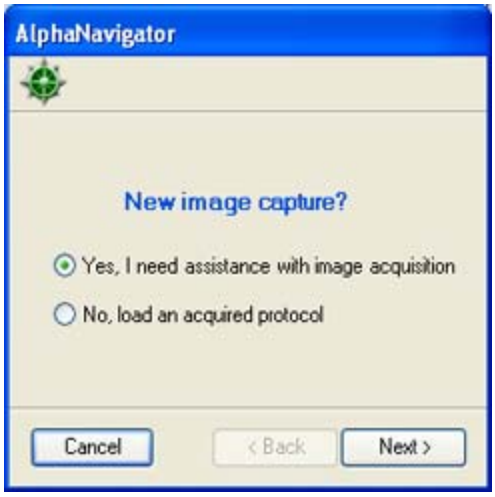
Figure 2.1 AlphaView screen, showing the image area and display controls

Basic Imaging Functions

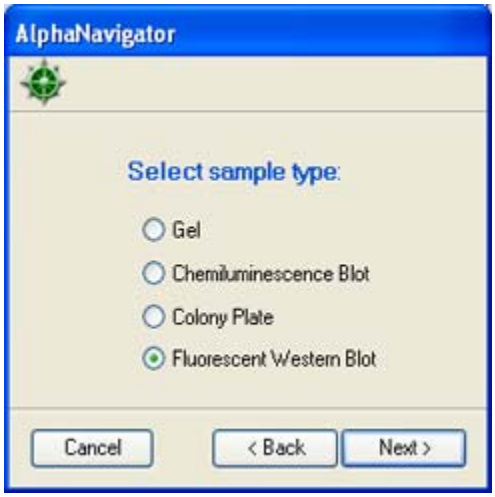
AlphaView software has four (4) main control windows for all image acquisition, contrast adjustment, enhancement, and analysis functions:

Acquiring an image - using the Navigator

 Clicking the Navigator toolbar icon will guide the user through commonly used applications, in this example the “Ethidium Bromide” application is demonstrated.



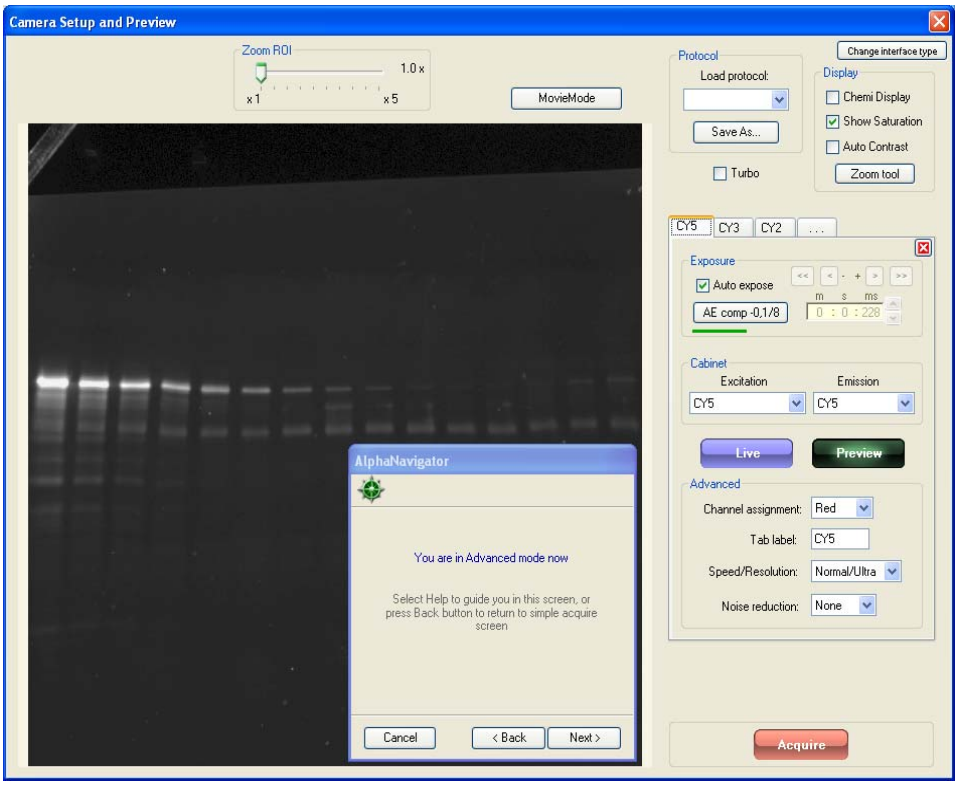
Selecting the “Yes, …” option:



Selecting the Gel option:

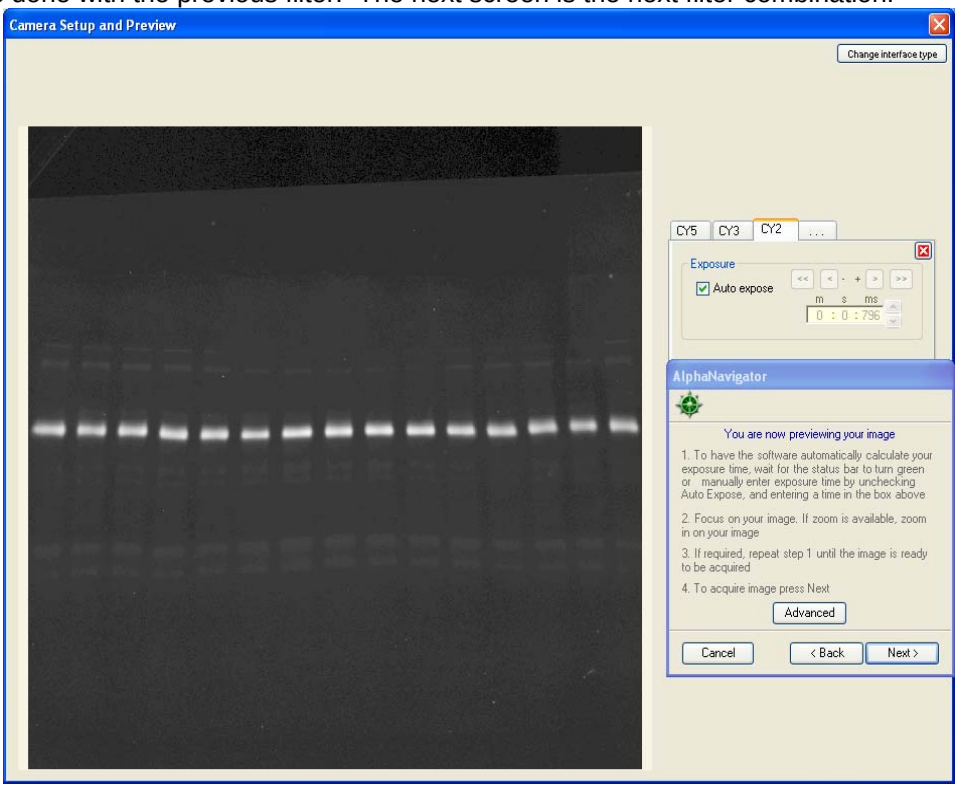
Select any of the filter combination –Cy5, Cy3, and C2 are selected here for channels 1, 2, and 3 respectively.

The first channel (Cy5) is displayed, user can access the advanced mode, or click next for the next filter combination. The next screen is advanced mode for the Cy5 filter combination, in this mode the user can modify any of the controls, clicks Next to go to the next filter combination, or back to the current filter combination preview mode.

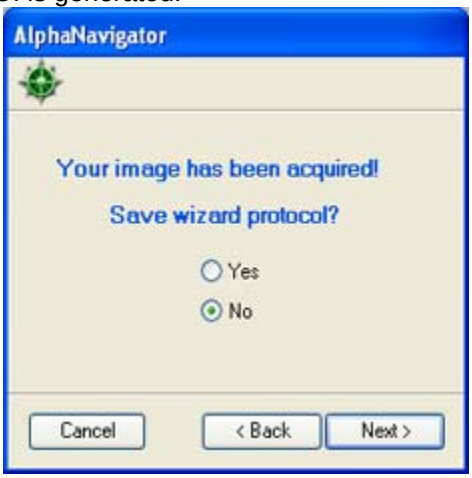


The next screen is next filer combination (Cy3):

The user has the same options as with the first filter, advanced mode, or next filter combination – same as done with the previous filter. The next screen is the next filter combination.



The user has the option to use advanced mode, back, or next to acquire, after the acquisition is complete, the save protocol UI is generated:



Selecting the Yes option and clicking Next> will give a save protocol UI. The protocol will be added to the list of existing protocols.

Selecting the No option and clicking Next>, will generate the save image UI:

Selecting the Yes option and the clicking the Finish button will generate the save image UI and exit the Navigator.
Selecting the No option, will close the Navigator.

The next time the Navigator is run, the user can run the saved protocol:

Selecting the No option, the following screen is generated with the last saved protocol selected:

The user can browse for other saved protocols by clicking on the "  " button, a list of existing protocols will be displayed:

Selecting the protocol will go to the preview screen for user to verify setting or acquire.

Camera Setup and Preview Window

Clicking the Camera Acquire Icon launches the Camera Setup & Preview window.

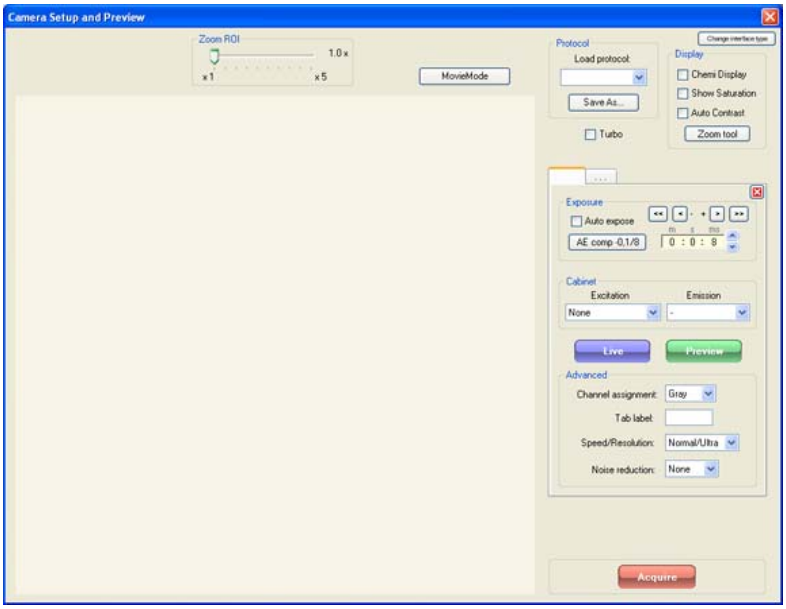


Figure 2.2 Camera setup and preview window

This window provides camera exposure control, sensitivity/resolution control, lighting/filter wheel position control, contrast display options, and cabinet door status. The active imaging channels are laid out in a tabbed architecture. One tab for each channel that contains the complete acquisition configuration for that channel.

Live

This window opens in Live control mode (blue button). Live control mode provides near-real time readout for easy sample positioning and optics adjustments. While in focus, select the lighting, filter, and binning options appropriate for your sample. You can then in on your sample, select an aperture setting, and focus on your image.

Preview

Clicking the green Preview box allows user to preview what your final image would look like, while still allowing you to make small adjustments to the options you select in focus.

Acquire

Select Acquire Image to capture you final image. You will then be able to save, print or analyze your image.

Movie Mode

Select Movie mode button allows user to acquire images in series, utilizing different imaging times, resolution modes and noise reduction settings. Used primarily for Chemiluminescent detection. See figure 1.24.

Auto Expose

FluorChem Q features an auto expose function for ease of use. The Camera Setup & Preview window includes a check box to activate the auto expose function as well as a button labeled Auto Expose Setup to configure the function.

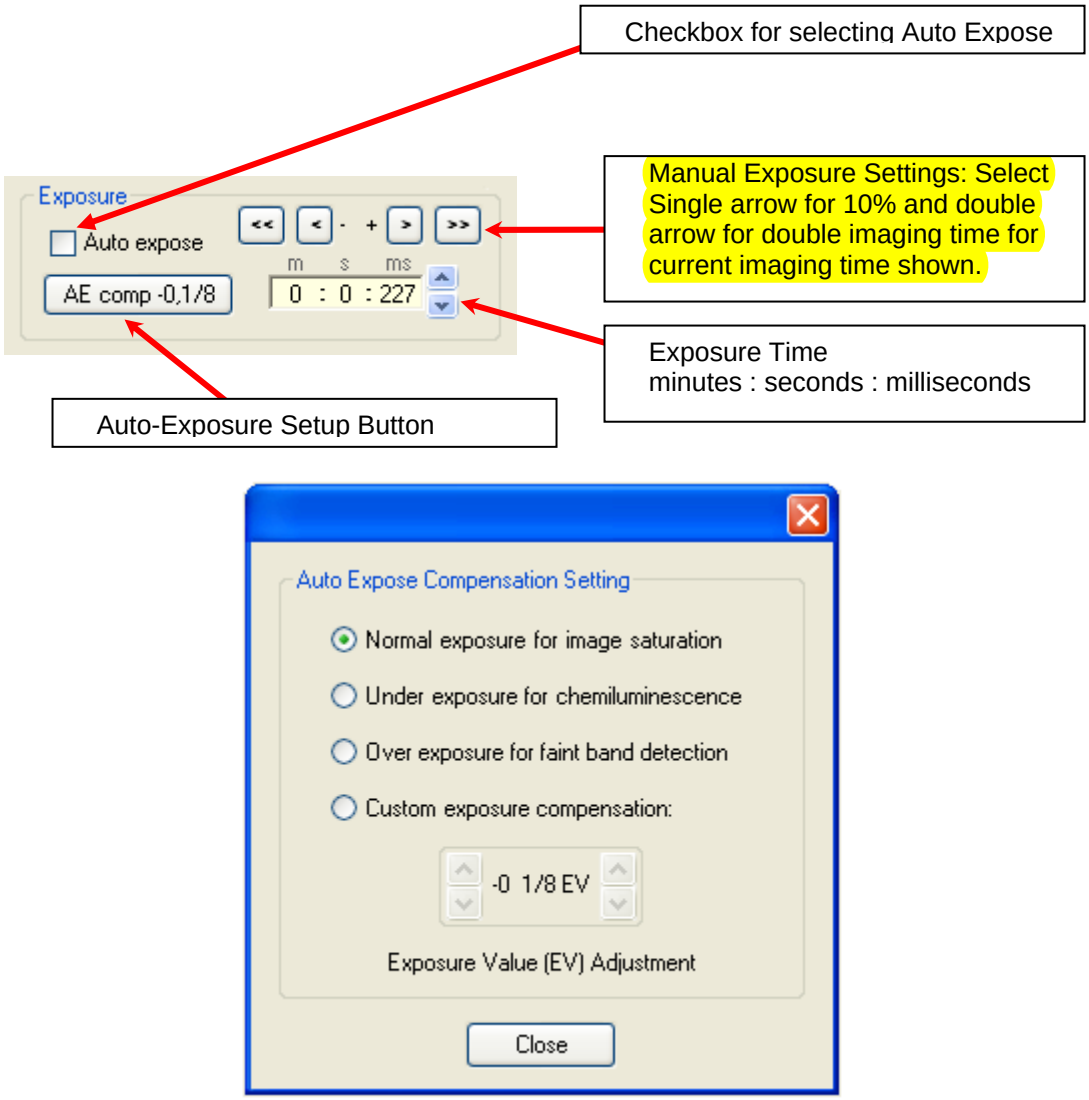


Figure 2.3 Auto Expose

Clicking the Auto Expose Setup button launches the Auto Exposure Compensation Setting window. This window offers four options:

- Normal Exposure for image saturation. This setting is ideal for normal Colorimetric and Fluorescent imaging.
- Under exposure for Chemiluminescence
- Over exposure for faint band detection.
- Custom exposure compensation. This setting allows the user to define the Exposure Value (EV). The left set of up/down arrow buttons change the EV value by whole unit intervals while the right set of up/down arrow buttons change the EV value by 1/8 unit intervals.

Auto Exposure works in both Preview and Acquire Image modes. The AlphaView™ software calculates the correct exposure time for the current situation. In Expose Preview mode, a status bar appears above the Auto Expose interface. In Acquire Image mode, a status window featuring a colored Auto Expose status bar for image acquisition appears. The status bar indicates the current status of the calculation. The status bar changes from red to yellow to blue to green to indicate progress. When it reaches green, the AlphaView™ software has calculated the correct exposure time. For exposure time calculations with saturated pixels in the image area, the status bar changes to pink to indicate the issue.

In Acquire Image mode, a green status bar indicates that the image is acquired. After achieving a green status, the new image loads and the Camera Setup & Preview window closes. In Expose Preview mode, the system continues repeated expose using the calculated exposure time until a different mode is selected.

Switching from Expose Preview mode to Acquire Image mode does not reset the calculation. If the system changes from Expose Preview mode to Acquire Image mode after calculating the correct exposure time in Expose Preview mode, images are acquired using the last calculated exposure time. Similarly, if a different sensitivity (binning) mode is selected after finishing the exposure calculation, the calculation is quickly converted and no recalculation is required.

The Exposure Value (EV) unit is the same as used in film photography (also called a “stop”). +1 EV will double the exposure time while –1 EV with halve the exposure time. The general exposure time using EV is found by the following formula:

$$\left(EVCompensatedTime\right) = Time * 2^{EV}$$

Where Time is the typical exposure time for the sample under test to reach full exposure as determined by the auto exposure tool.

Setting Exposure Time Without Auto Expose

Once the sample is positioned and the lens is properly focused, close the MultiImage light cabinet door and check to ensure that the appropriate illumination source is turned on. In addition, check to ensure that the cabinet door indicator in the Cabinet Control software interface indicates Closed.

Uncheck the Auto Expose option.

Click on the green Expose Preview button and select the desired exposure time in to configure the system for the desired image intensity quality. Individual adjustments for milliseconds, seconds, minutes, and hours are available. The following list describes ideal exposure settings for different applications:

- For most white-light applications, using a 50ms exposure is sufficient. Final aperture adjustments can be made to optimize the image quality.

- For UV fluorescence applications, using an exposure range between 8ms and four seconds is sufficient. The aperture should be completely open when performing any adjustments. Selection Show Saturation is recommended for these applications.

- For low-light applications, such as chemiluminescence, a longer exposure time may be appropriate. In these cases, push the red Acquire Image button to directly acquire the

image upon setting the desired exposure time. If a good exposure time is unknown, an alternative method is to utilize the Auto Expose feature configured to super sensitivity. Auto Expose image generation – with signal intensity showing – generally completes in less than three minutes. After Auto Expose image generation, a different sensitivity (binning) mode can be selected to generate a better resolution image. For further images, the software automatically calculates the new exposure time.

NOTE: When the system switches to Expose Preview mode, the image may flash or change brightness due to camera photon collection from the image over a longer period of time prior to sending the image to the computer's display readout.

Light Source & Filter Controls

The mechanical controls on the cabinet and the linked FluorChem Q software virtual cabinet control interface (labeled as Cabinet Controls) provide near-real time synchronization and updating of light source and filter selection. Selecting a desired option via the software interface produces the same result as using the physical controls on the cabinet.

NOTE: A slight delay occurs upon pressing the button and activation of a light source.

Standard lighting choices include:

Transillumination White:	protein gels, autorads, film, plates, flasks
Transillumination UV:	fluorescent gels such EtBr, SYPRO Red, SYBR Safe, etc.
Reflective White:	colorimetric blots and membranes
Reflective UV (optional):	SYBR green, TLC plates, and Chemifluorescence
ChromaLight (optional):	GFP, Fluorescein, and SYBR green

Filters selection is linked to appropriate sample visualization.

Filter options include:

Filter Position #1:	Empty Position for Chemiluminescence detection
Filter Position #2:	Ethidium Bromide, colorimetric stains, film, SYPRO Orange (595nm)
Filter Position #3:	CY2, Fluorescein, SYBR Gold (520nm), SYBR Safe (530nm)
Filter Position #4:	CY3, SYPRO Red, Texas Red (630nm)
Filter Position #5: OR Empty Position	CY5**Filter Position #6(optional):** Hoechst Blue (460nm)

NOTE 1: Each Filter has an approximate bandwidth of +/- 40nm to allow for use with other fluorescence stains as they are developed. For custom applications, please contact Alpha Innotech directly to discuss custom filter design for specific applications.

NOTE 2: If exposure time is less than 100 ms, yet signal is strong, close the aperture by several f/ stops.

Turbo Modes and Speed/Resolution Settings

FluorChem Q system offers different speed and resolution settings to allow image acquisition across a variety of needs. Tweaking resolution and speed settings allow image acquisition to occur as quickly as possible, with the highest resolution possible, or somewhere in between. Available acquisition modes include 5 binning modes and 2 Turbo modes.

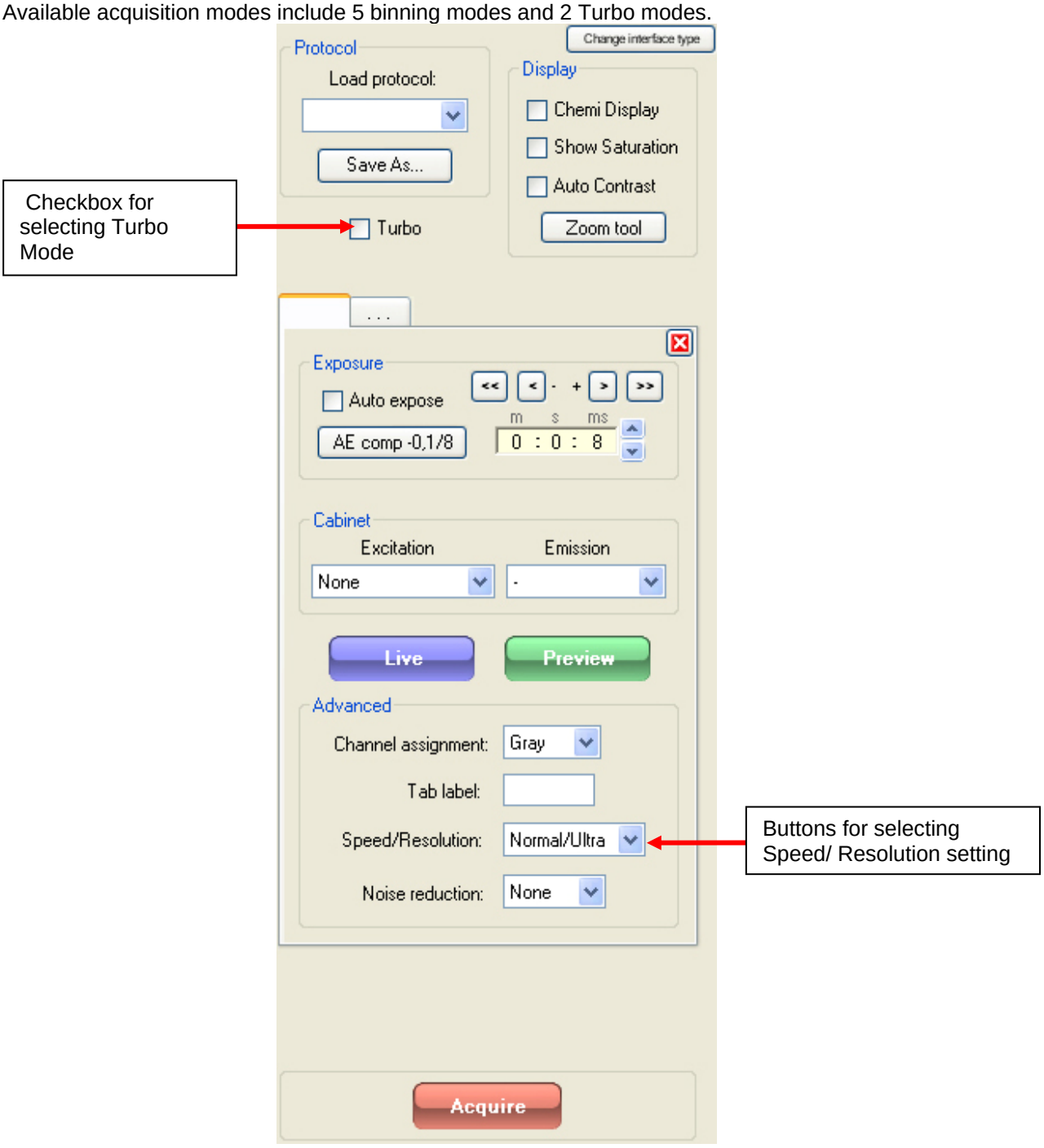


Figure 2.4 Turbo Modes and Speed/Resolution Settings

The camera modes are selected by the Turbo Mode check box. When the Turbo Mode checkbox is unchecked the camera operates in Normal mode. Normal Mode is useful for those imaging applications that require wide dynamic and quantification of low signal intensities. When the Turbo mode checkbox is checked the camera operates in Turbo Mode. Turbo Mode is used for the applications that require fast image acquisition to quickly visualize samples even those with low signal intensities. Both Normal and Turbo mode can be used with each of five binning modes and auto expose functions to optimize image acquisition speed and for fluorescence and chemiluminescence applications

Speed/Resolution Settings

Normal /Ultra
Recommended for Fluorescence and Colorimetric Imaging, this mode captures the image using the CCD sensors full 2048x2048 pixel resolution to generate highest quality, vibrant images that are ready for quantitative analysis and publication.

Medium /High
Recommended for Chemiluminescence, this mode captures images with a 2x2 pixel bin to decrease the required exposure time while maintaining a high resolution (1024x1024) image .This mode decreases exposure times from full resolution images by approximately a factor of four.

High /Medium
Recommended for Chemiluminescence, this mode captures images with a 3x3 pixel bin (683x683 pixels) with decreased image acquisition time and medium resolution. This mode decreases exposure times from full resolution images by approximately a factor of nine

Fast/Low
Recommended for Chemiluminescence, this mode captures images with a 4x4 pixel bin (512x 512 pixels) Similar to Super Speed this mode decrease exposure time but with a tradeoff in resolution. This mode decreases exposure times from full resolution images by approximately a factor of sixteen

Super Speed
Recommended for Chemiluminescence, this mode captures the image performing an 8x8 pixel bin (256 x 256 pixels). This mode significantly decreases the exposure time required for acquiring a long chemiluminescent exposure. This mode's resolution is set to 256x256 pixels and is not recommended for precise quantitative analysis or publication. Super Speed is ideal for Expose Preview functionality when using auto-expose to determine the chemiluminescent blot signal level. It is also useful for rapid visualization of signals for a qualitative assessment of the sample.

Gray Scale Optimization for Saturation and Contrast Displays

The following figure illustrates the check boxes available for modifying the displayed image.

Any images set for analysis must not be over-exposed (too light) or under-exposed (too dark) for maximum clarity. Using Show Saturation displays the image areas assigned to each end of the gray scale spectrum. After inspecting the image's saturation, the imaging controls can be adjusted accordingly to optimize the image**.**

The Show Saturation checkbox (found below the camera control functions) allows the user to access the Saturation Palette during image acquisition. The Saturation Palette is a modified gray scale palette in which green replaces black (gray level 0), and red replaces white (gray level 255, 4,095, or 65,535). With this palette, the image's over- and under-exposed areas appear as green or red, while areas within the linear range of the CCD chip appear in gray scale.

During image acquisition, it is critical to note the image's regions that appear red or green. The exposure time and the camera aperture can be adjusted to minimize the amount of red and green in the image area. Eliminating red and green in the actual sample area is especially critical for images that will be quantified. The saturation view can be turned off by unchecking Show Saturation once the red and green areas have been minimized and/or eliminated

The other two selections (Chemi Display and Auto Contrast) act as visualization tools designed to enhance contrast and provide flexibility image viewing. Auto Contrast displays the image with automatic black, white, and gamma adjustments according to the image histogram information (black/white levels). Chemi Display utilizes both auto contrast and reverse functions as well as providing gamma adjustments and is intended for use with chemiluminescent samples. These options are also available in the Movie Mode.

NOTE: These Contrast Display options are only visualization tools and do not change any image data acquired by the CCD camera.

Zoom ROI

Zoom ROI is a tool designed for use with fast or fixed focal length lenses. These lenses typically have lower F-stop numbers than zoom lenses but do not have zoom capabilities. Fast lenses are most commonly used to decrease exposure times in chemiluminescence applications. However, they can also be used for other applications, such as fluorescent gels. Since fixed focal length lenses do not have zoom rings, adjusting an acquired image's region of interest is possible using the AlphaView™ software interface.

When the sample's region of interest is smaller than the acquisition window's and the system is not equipped with a zoom lens, the zoom ROI tool provides an ideal workaround. The Zoom ROI tool allows for software-based zooming without having to switch lenses. To use the Zoom ROI tool, focus on the image, then position the Zoom ROI slider until the region of interest occupies the preview window's entire image area. Use the Expose Preview and Acquire Image buttons to configure and acquire the final image. The Zoom ROI tool eliminates the need for cropping an image down to a desired size post-acquisition.

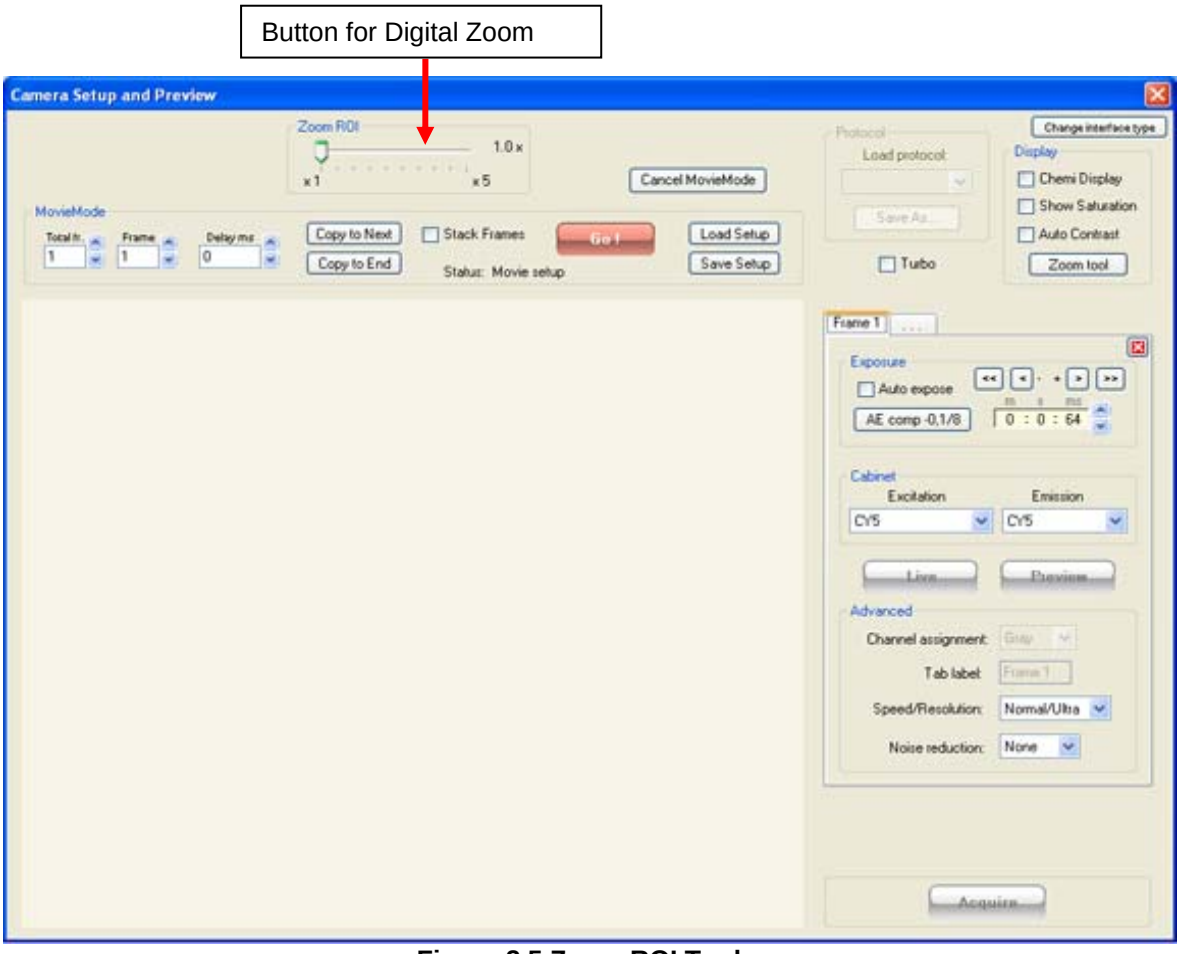


Figure 2.5 Zoom ROI Tool

Saving Images

Once a satisfactory image has been captured, it should be saved as an original file for safe archiving. Use the Save As function in the File menu or click on the Save or Save As icons.

Movie Mode

If kinetic, multiplex, color, or chemiluminescence experiments require the system to automatically capture several images at preset exposure times, preset time delay between images, preset lighting sources, or preset filter choices, AlphaView™ Movie Mode should be used. Clicking the MOVIE box in the ToolBox's Enhancement Tools tab launches the Movie Mode controls. Movie Mode setup is also accessible in the Camera Setup & Preview window. To launch the setup screen, click the Movie Mode button in the Camera Setup & Preview window.

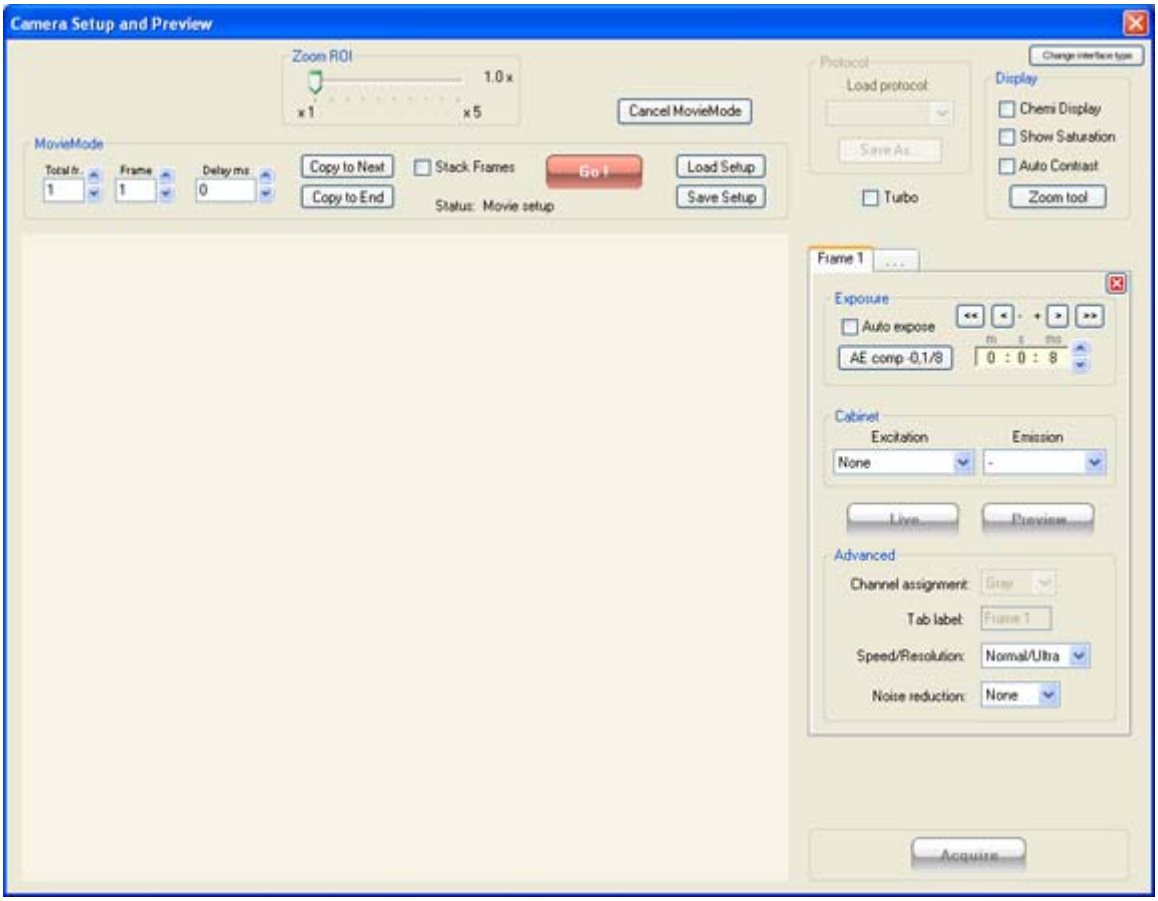


Figure 2.6 Movie Mode Setup

The Total Frames field sets the total number of individual frames in the movie. Each movie can capture maximum of 50 frames (images) and a minimum of one frame.

The Frame field selects the active frame currently being used for condition customization. For example, if the movie uses three images, selecting Frame 1 allows for lighting and filter setup of the movie's first frame; selecting Frame 2 allows for setup of the movie's second frame. All

frames can be adjusted manually using this technique, or specific lighting conditions can be copied to the next frame using the Copy To Next button. Copy To Next copies all settings from the previous frame to the current frame.

For chemiluminescence imaging, all lighting is typically shut off and the filter wheel is positioned for the chemiluminescence position across all frames. In these situations, the exposure time is the only variable that changes from frame to frame. Copy To Next is a useful tool that optimizes setup time.

The Delay field configures a predetermined delay between captured imaged for kinetic experiments. The default Delay is set for the shortest possible delay (19ms), but can be configured up to 50 minutes between images.

Once the movie setup is properly configured, click the Go button to begin the movie process. The system begins processing image acquisitions for each frame. When completed, the movie setup box disappears and the Toolbox window automatically configures to the Movie tools. This allows for movie playback, saving, loading, or new movie recording.

The movie display buttons offer the following functionality:

REC	Launches the Camera Setup and Preview window configured to Movie Mode.
PLAY	Displays the movie in a continuous loop.
STOP	Stops the movie at the current frame display.
PAUSE	Pauses movie playback at a user defined image.
SAVE	Images are automatically opened after completion of movie mode setup.

Save/Load Setup Acquisition

Individual movie parameters can be saved for future use. The Save Setup and Load Setup buttons save and load all Movie Mode setup parameters. This data is saved in files using the MVF format.

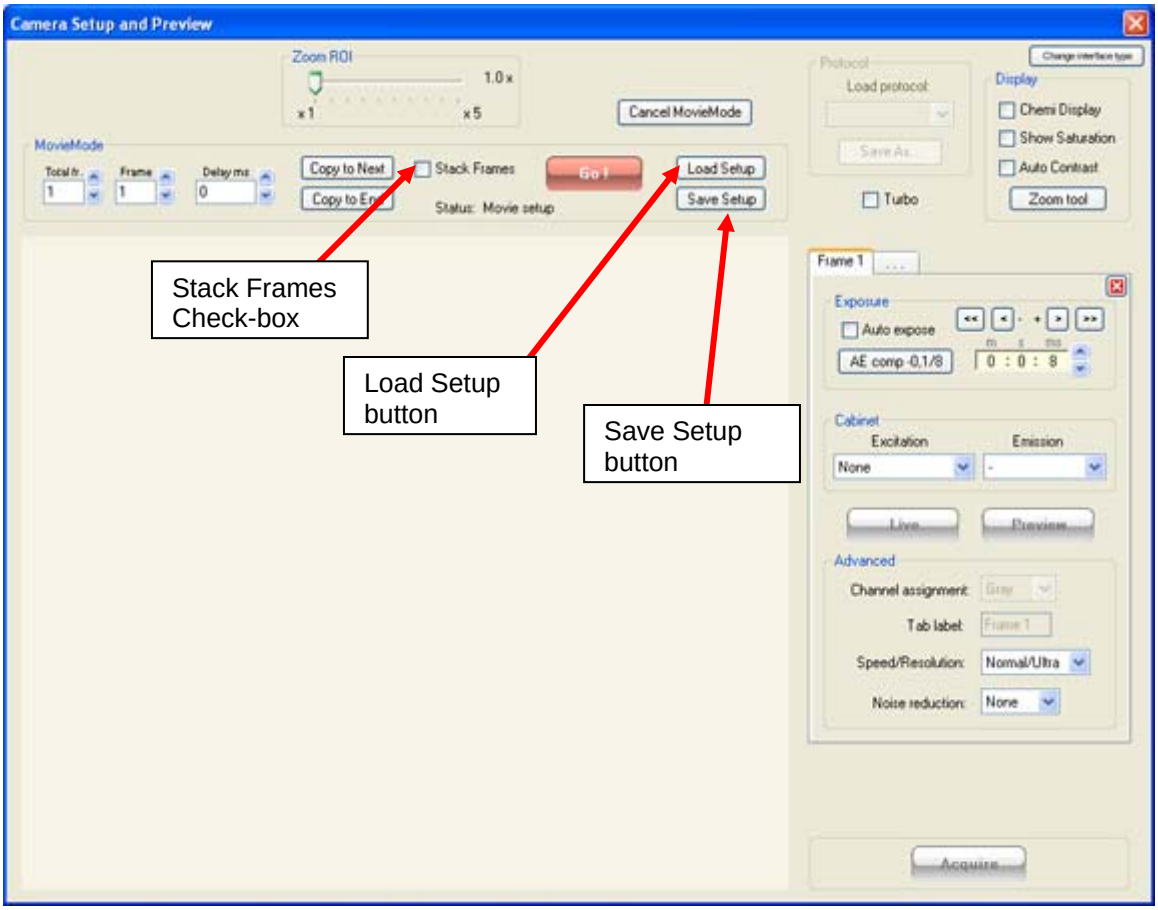


Figure 2.7 Movie Mode: Load/Save Setup

Frame Stacking

The Movie Mode configuration of the Camera Setup & Preview window provides an option for stacking frames. If this selection is used during image acquisition, Stack Frames uses previous exposure information to sequentially add images to one another. Normal Sequence does not perform this addition.

NOTE: Stacking frames increases the noise level in acquired images.

Sample Case

Sample case:

Capture 5 frames at 1-5 sec exposure for total time exposure time of 15 sec

Display after summation of following frames:

Frame 1 = Image (1 sec exp time)

Frame 2 = Frame1 + Image (2 sec exp time)

Frame 3 = Frame2 + Image (3 sec exp time)

Frame 4 = Frame3 + Image (4 sec exp time)

Frame 5 = Frame4 + Image (5 sec exp time)

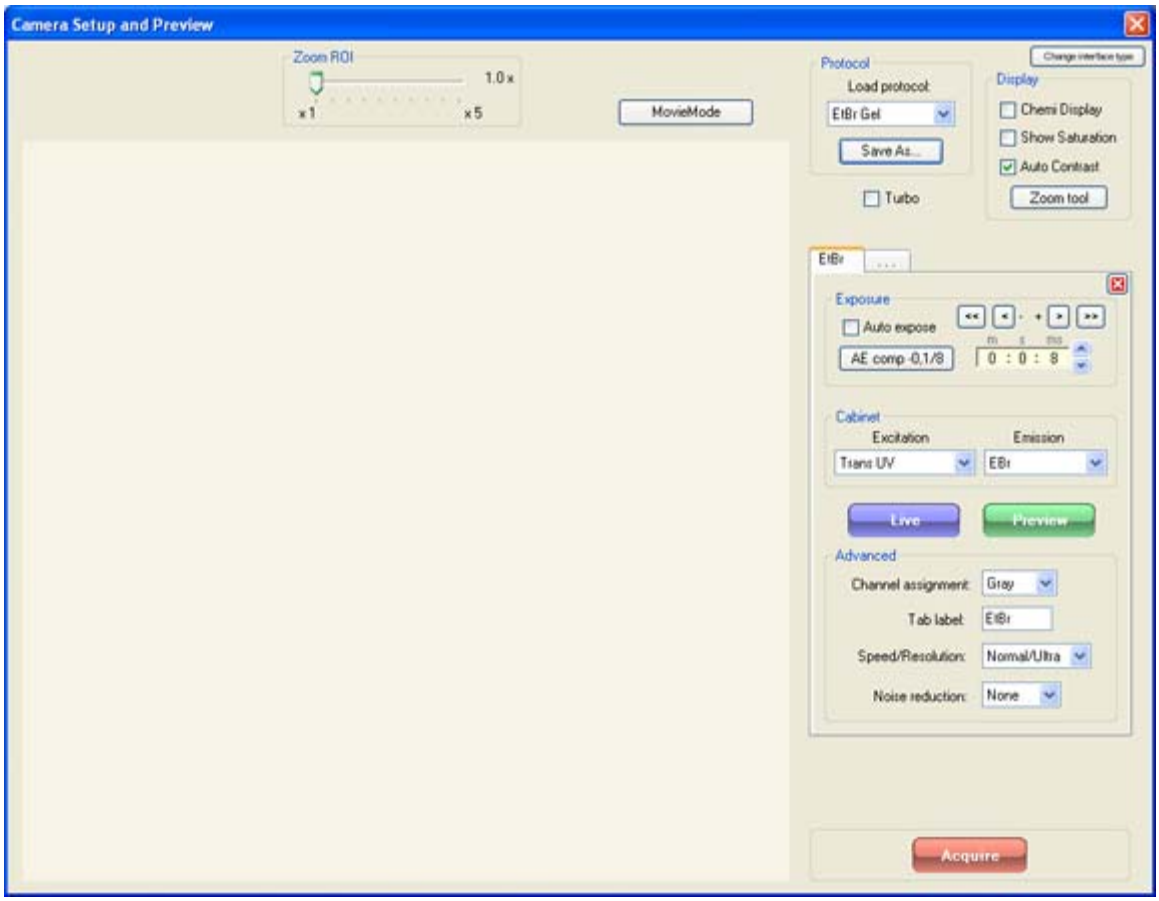
Automatic Image Capture (AIC) Software

For molecular biology researchers engaged in high throughput and particularly high volume work involving repetitive assays, Automatic Image Capture software is available. This system automatically adjusts lens focus, filter settings (for fluorescent imaging), light settings and exposure for perfect images. All of this with just one click--making the acquisition of images faster and more convenient than standard imaging software. Note:
AIC requires motorized zoom optics.

Designing a User Protocol

To utilize the single click feature of Automatic Image Capture a protocol specific to your sample must be defined and saved. Click the Acquire button to reach the Camera Setup and Preview Window.

All acquisition parameters that can be set by the user are available in the Camera Setup & Preview window and can be saved as a user protocol. Below is an example of the design of a protocol for imaging DNA gels stained with Ethidium Bromide.



Next, select **Save As…** in the Protocol Tool to save your customized protocol.

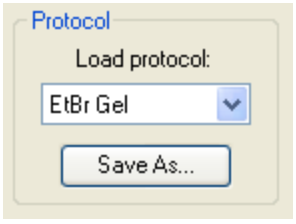


Figure 2.8 Save/Load Acquisition Protocol

The Save/Load Acquisition Protocol Window will appear, and allow to you Save and name your protocol.

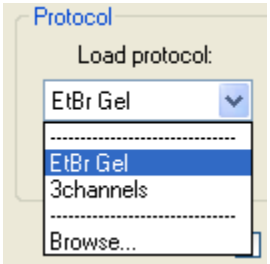


Figure 2.9 Save/Load Acquisition Protocol

This window is also useful for loading a previously saved protocol to make small modifications, or delete existing protocols.

Auto Image Capture (AIC)

User-customized protocols can be recalled for single click image capture using the AlphaView™ software's Auto Image Capture feature. First, load the protocol of interest by clicking on the drop down menu from the Auto Image Capture Icon.

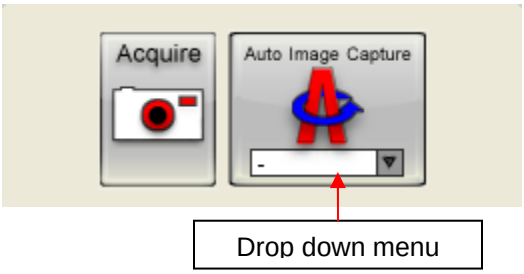


Figure 2.10 Auto Image Capture (AIC)

Once the protocol is selected, click on the Auto Image Capture button to begin the acquisition process.

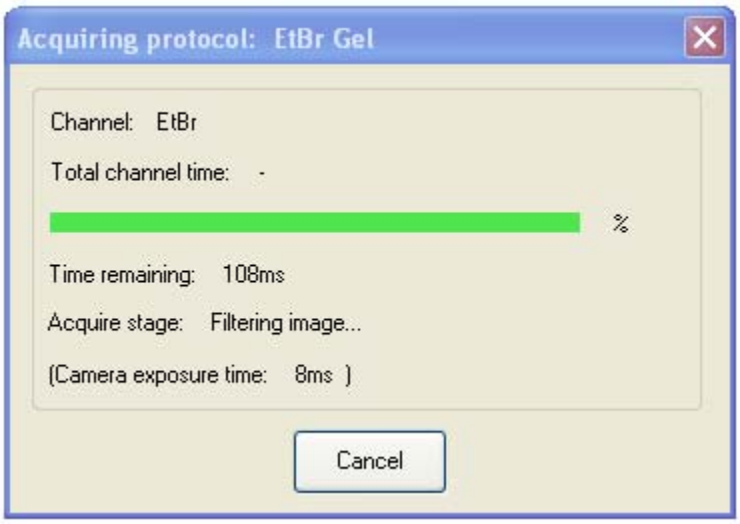


Figure 2.11 AIC Status Window

Once the Auto Image Capture process is initiated, a pop-up window, labeled "Acquiring protocol: [protocol name]", is displayed to indicate the status of the acquisition process. This progress window shows the current operation status and overall imaging progress.

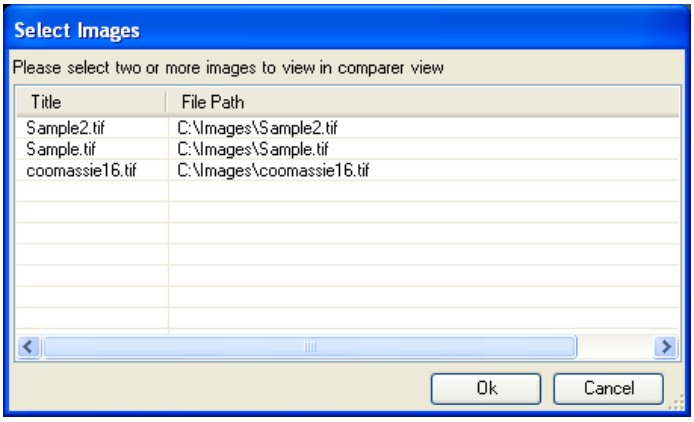
NOTE: The images displayed in the Acquiring Protocol windows are animations to display status and do not reflect upon the image being acquired.

Compare View

Two or more images can be opened in compare view. To open compare view, click on the Compare View icon.

Compare View toolbar button

If only two images are opened in the tab view, images will directly open in compare view. If more than two images are opened in tab view then a dialog box pops up, through which image can be selected to open in compare view.



If two images of same size are opened in compare view then synchronize checkbox, in status bar, will be available to use. By activating synchronize function scroll and zoom can be applied on both images together.

To switch back from compare view to tab view simply clicks on the Compare View icon.

Another way is by drag one image tab to see the docking options as shown in the following figure

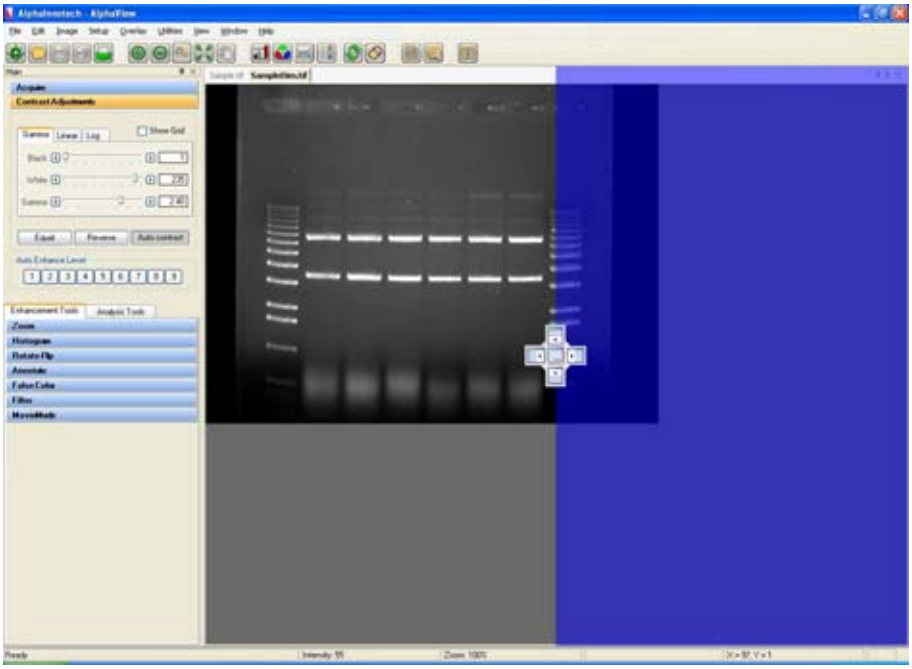


Figure 2.12 How to enter Compare View

After selecting appropriate place, when you drop the image tab, compare view will be opened.

Figure 2.13 Compare View

AlphaView™ System Quick Guide

Note: This is intended as a quick reference guide for acquisition. For more detailed information on the individual features reference section 1.6 of this manual.

1. Power on the system:
    a. Turn on the computer, monitor, and optional printer.
    b. After the computer has finished loading the Windows desktop, turn on the power to the cabinet.
    c. The AlphaView™ software is activated by double clicking on the AlphaView™ icon.

2. Positioning and focusing on the sample:
    a. In the Tool Bar, select the Acquire icon to activate the image acquisition software features.
    b. In the Camera Setup & Preview window, click the Live button.
    c. Open the door to the cabinet and position your sample on the preferred illumination source. Fluorescence samples that require epi or transillumination of UV energy should be placed on the purple UV filter glass. For colorimetric samples such as protein gels, films, or blots, use the fold-down white light table for your sample. For chemiluminescence use the fold-down white light table, or the adjustable tray if used in conjunction with the fast lens.
    d. Manually open the aperture on the camera lens all the way (select the smallest number on the side of the lens – f/0.95).
    e. With the door still open to allow light to enter into the cabinet, use the monitor's real-time (**LIVE**) readout display to position and focus your sample in the middle of the preview image.
    f. Focus on the object by adjusting the top knurled knob on the lens. For fluorescence and chemiluminescence, the door to the cabinet can be used to adjust the amount of light that enters into the cabinet if there is too much light to obtain a good focus setting. Do not adjust the aperture to do this. For colorimetric samples, it is necessary to decrease the aperture to acquire the image.
    NOTE: Business cards and other pieces of paper with small text often are the easiest objects to obtain optimal focus settings.

3. Capturing a bright sample like fluorescently labeled gels, colorimetric samples and film:
    a. Close the cabinet door.
    b. Choose the appropriate optical filter for your sample type:
        - Position #1 for colorimetric gels and film (no filter).
        - Position #2 for Ethidium Bromide gels.
        - Positions #3-6 for other fluorescently labeled gels (optional filters).
    c. Select the illumination source (LED, UV or white light) using the touch panel or software controls.
    d. Select the green Preview button.
    e. Check Show Saturation.
    f. Check Auto Expose.
    g. Select the Normal/Ultra resolution setting.
    h. Once the image in expose preview does not contain any saturation (red false color palette for white bands, green for dark bands) select **Acquire**. The exposure bar will turn green when this is complete. If the exposure bar is pink in color, saturation is still present in the image. For extremely bright images, particularly in white light applications, it may

be necessary to reduce the aperture setting until the saturation is removed from the image.

4. Capturing a low light image (like chemiluminescence)
    a. Focus with the lens aperture wide open (f/0.95).
    b. Close the cabinet door (there should not be any lights on in the cabinet).
    c. Choose Position #1 in the filter wheel for no filter.
    d. Select the green Expose Preview button.
    e. Check Show Saturation.
    f. Check Chemi Display.
    g. Check Auto Expose.
    h. Select the Super Speed resolution setting.
    i. Wait for the exposure bar to indicate that the proper exposure time has been found and that there is no saturation in the image - the exposure bar will turn green when this is complete. If the exposure bar is pink in color, saturation is still present in the image.
    j. Select Medium/High or Normal/Ultra resolution and uncheck Auto Expose.
    k. Click Acquire Image.

    NOTE: The exposure time will vary depending on which resolution setting is selected in step 4.h. If the exposure time calculated by the auto expose setting is too long, it is possible to use the Fast/Low or High/Medium setting instead. Alternatively, if the exposure time is short enough, Normal/High may be selected for a full resolution (4.2 million pixels) chemiluminescent image.

5. Save the original image
    a. Select Save Image in the File menu or click on the SAVE or SAVE AS icon in the Tool Bar.
    b. Enter a file name and select the directory to save image (the directory path should be less than 100 characters).
    c. Specify the file format (TIF, BMP, PCX, MAC, color TGA).
    d. Click OK to save the file.

6. Enhance the display (optional)
    a. Adjust the black, white and gamma levels by moving the slider bars at the right of the image in the Contrast Adjust window, or select Auto Contrast.
    b. Apply the digital filters found in the Tool Box under the Enhancement and Filters (to stop a filter, hit any key on the keyboard; to reverse the effects of a filter, click Undo).
    c. Add text, boxes, arrows, etc. to the image using the annotation tools in the Tool Box under Enhancement and Annotate.

7. Print the image using the PRINT button in the Tool Bar or the pull-down File menu option. Analyze the sample using the analysis features in Tool Box (ANALYSIS for quantitative analysis).

Contrast Adjustment

The Contrast Adjustment window allows for the best visualization possible of a sample utilizing the black, white, and gamma adjustments, as well as, image reverse and auto contrast.

The image on the screen is made up of picture elements (pixels) in an array. Each pixel is assigned a brightness (or a gray scale value) level between black and white. A very bright image has most of its pixels registering high gray level values and conversely, a very dark image has most pixels registering low gray level values (approaching zero).

The distribution of these gray values to the image is determined by the Contrast Adjustment Controls. These controls regulate the Black level, White level, and Gamma setting (brightness linearity), allowing adjustment of the display to obtain the best image possible.

Note: These enhancement features modify the image display on the monitor only, and do not change the original quantitative data.

AlphaView software can also import RGB color images. The AlphaView Software automatically detects this process and the Contrast Adjustment tools are configured for color image adjustments.

An image can be enhanced using these tools and then saved as a Modified file for publications. However, to preserve the original image information, it is recommended that the file be saved as a different file name when using the save modified.

Using the Contrast Adjustment Tools for Grayscale Images

There are three sliding scales found in the image control area to the right of the image. Below each scale is a box displaying a number that corresponds to the position of the slider. By adjusting these sliding scales, the image display can be optimized.

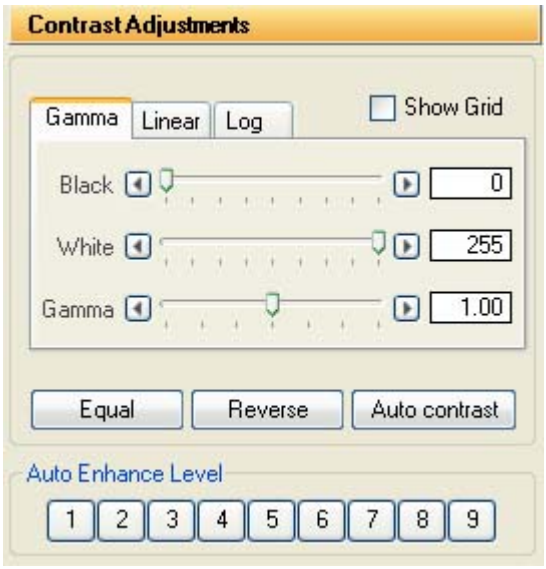


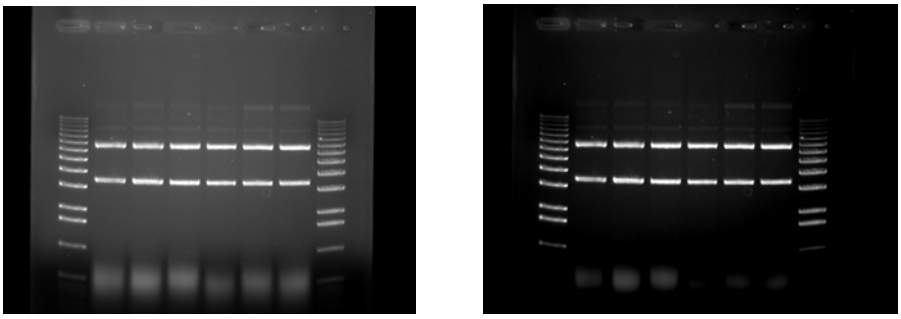
Figure 2.14 Contrast Adjustment Tool

Imaging Display Tools: Black Level, White Level,
Gamma Setting with B/W/G, Linear, Log, and Equalize options

To adjust any of these settings, place the cursor on the slider. Click and hold down the left mouse button while dragging the slider to a new setting. As the slider is moved along the scale, the image display is updated, along with the change in numeric value. The arrows above and below the scale bars can also be clicked to change the settings in single unit increments, or, the user may type in a specific unit.

Black Level Adjustment

The number beneath the Black Level scale corresponds to a gray level. There can be 256, 4095 or 65,536 possible gray levels depending on the system type. For the example below, an 8 bit image will be used with 256 total gray scale values. When the Black slider is at the very top of the scale, the number is 0. As the slider is moved downwards along the scale, the number increases and the image becomes progressively darker. This is because all pixels at the specified gray level and lower are shown on the screen as black pixels. If the slider is set to 0, all the pixels whose gray levels are at 0 are shown as black. If the setting is then changed to 60, all the pixels between 0 and 60 are shown as black and the image appears darker.

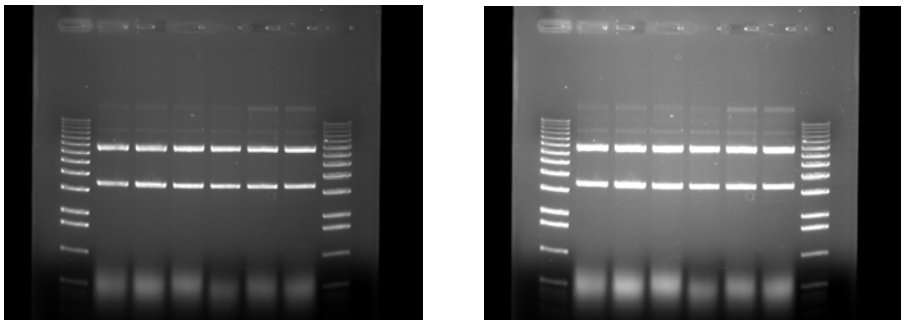


Black Level set at 0 Black Level set at 60

Figure 2.15 Black Level Adjustment example

White Level Adjustment

The number beneath the White Level scale also corresponds to a gray scale value. When the slider is at the very bottom of the scale, this number is 255. As the slider is moved upwards along the scale, the number decreases and the image becomes progressively lighter. This is because all pixels at the specified gray level value and above are shown on the screen as white pixels. For example, if the slider is set to 150, all the pixels between 150 and 255 are shown as white and the image appears lighter.



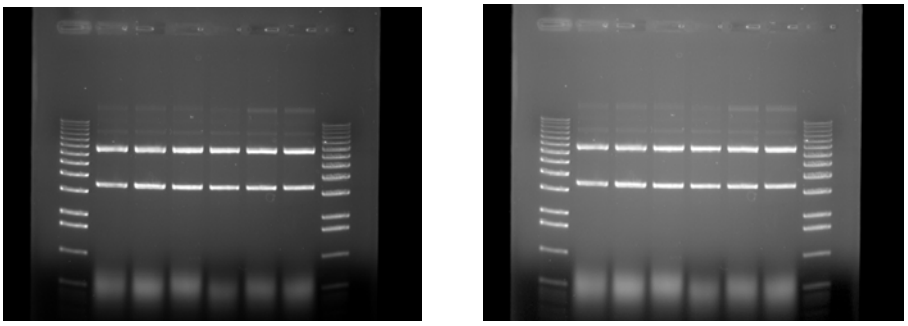
White Level set at 255 White Level set at 150

Figure 2.16 White Level Adjustment example

Gamma Setting Adjustment

Changing the Gamma setting affects the image brightness by adjusting the linearity of the image on the screen and printouts, but does not affect quantitative data.

The camera sees objects linearly while the human eye does not. When the Gamma setting is set to a value of 1, the image is displayed as the camera sees it. This, however, is different from what the human eye detects. By adjusting the Gamma setting, the user can make the image on the screen correspond to what is seen when he/she looks directly at the object. We recommend a Gamma setting of 0.55 for best visual representation.



Gamma set at 1.0 Gamma set at 0.55

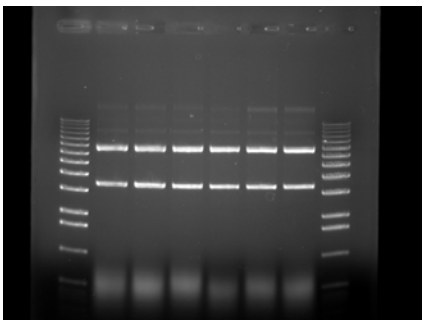
Figure 2.17 Gamma Setting Adjustment example

The Auto Contrast Selection

The Auto Contrast feature will automatically scale the black and white values of an image to more tightly fit the gray scale intensity profiles (histogram). This selection will use different black and white values for different images depending upon their unique histograms. A more dramatic visual change will take place for low light level images (such as chemiluminescence) where smaller portions of the histogram are used. This selection can be turned on or off and will adjust differently for each image.

The Reverse Button

The Reverse button inverts the gray levels of the displayed image, converting a positive image to negative, or vice versa. For instance, an image with black bands on a white background is converted into an image with white bands on a black background by simply clicking the Reverse button. Clicking the button a second time returns the image to its original form.


Original Image

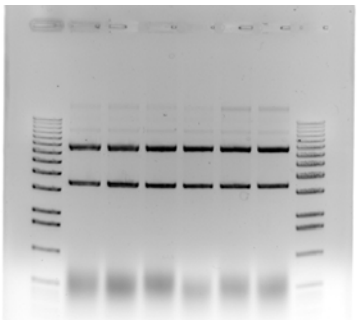
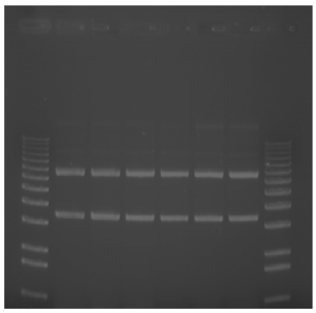

Reversed Image

Figure 2.18 Original and reversed Image

Note: Reversing an image changes the way it is displayed on the screen, but does not change the quantitative data. For example, the bands in the above gel have the same density, regardless of whether the gel is displayed as white bands on a black background or black bands on a light background. For information on reversing pixel values, see Invert in Chapters 4 and 5.

The Equal Button

The Equal button flattens the image to show all pixel values in the original image. Equal automatically adjusts the image display for maximum contrast which is beneficial for faint band detection.


Original Image

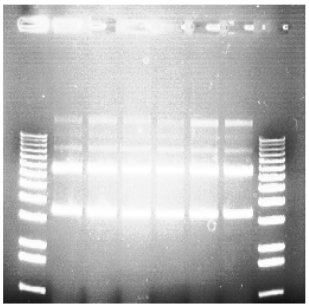
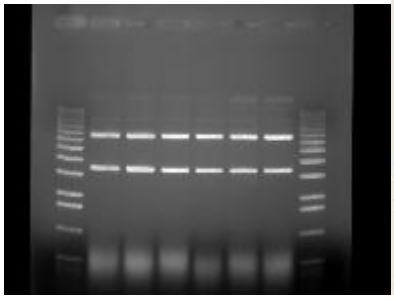

Equal Image

Figure 2.19 Original and Equal Image

Making Linear, Log, or Equal Adjustments

Original image of film with default BWG settings



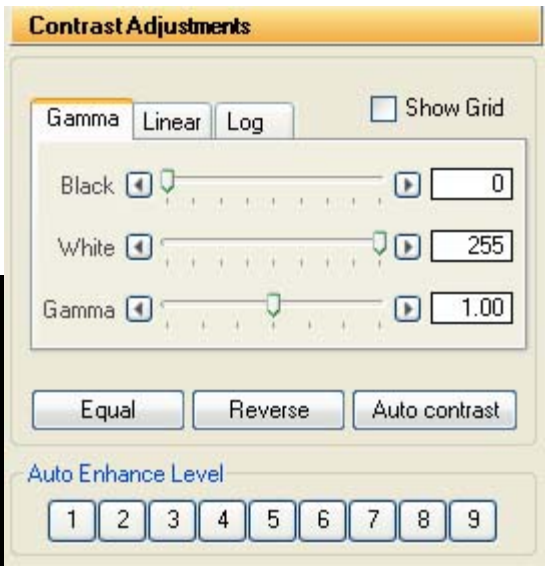
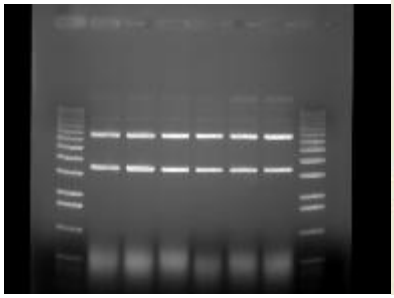


Image of film with linear Contrast Adjustments selected. Linear provides minimum and maximum adjustment tools from 0 to 100%. Linear stretches the grayscale range of the displayed image to the maximum system dynamic range of 0-65,535 grayscales.



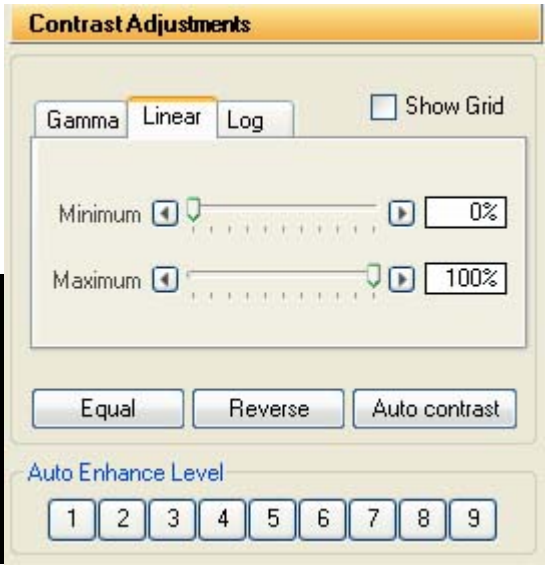
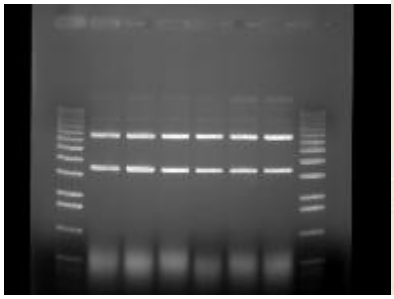


Image of film with log Contrast Adjustments selected
log provides minimum and maximum adjustment tools from 0 to 100%. Log performs a logarithmic adjustment to the grayscale range of the displayed image.



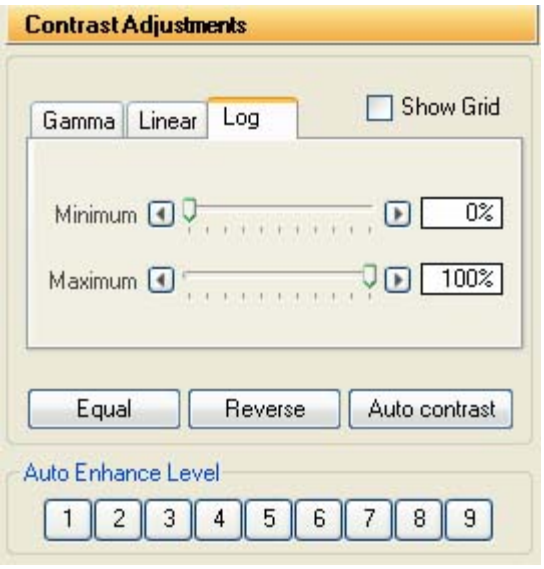
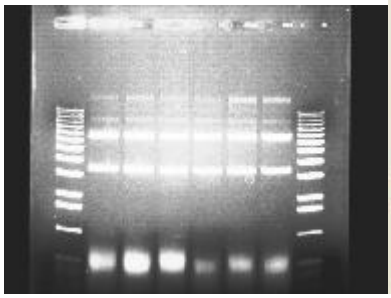
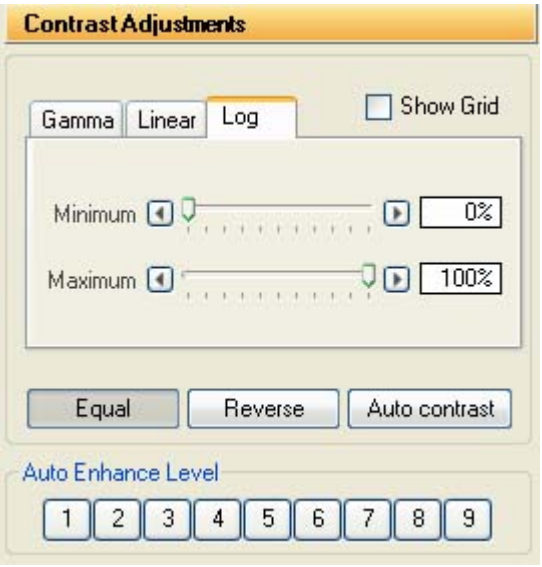


Image of film with equal Contrast Adjustment selected. Equal automatically adjusts the image display for maximum contrast which is beneficial for faint band detection.





Multicolor Image Display

Channel Viewer

Channel Viewer performs a preliminary review of a composite display of a multichannel image to view each channel separately in the context of the composite image. While each channel may also be displayed as a single channel using the Contrast adjustments window, the Channel viewer provides a convenient tool to explore the image in the composite display mode. Note: Channel viewer is only active when selecting a multichannel image.

Select **Channel Viewer** to open a display window capable of showing each channel independently. Hold down the left mouse button on the **Channel Viewer** window to select and drag the window to any position on the image. An average of each channels intensity (Red, Green and Blue) is displayed for the region in the window.

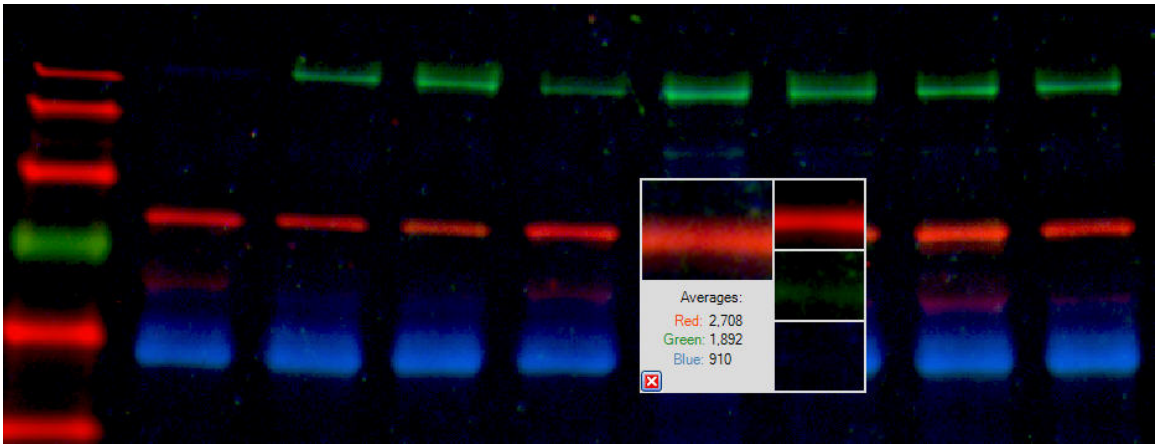


Figure 2.20 Channel Viewer

The Channel Viewer is useful for scanning a composite image. A region of the composite image is displayed along with a separate display of each underlying channel.

Contrast Adjustments

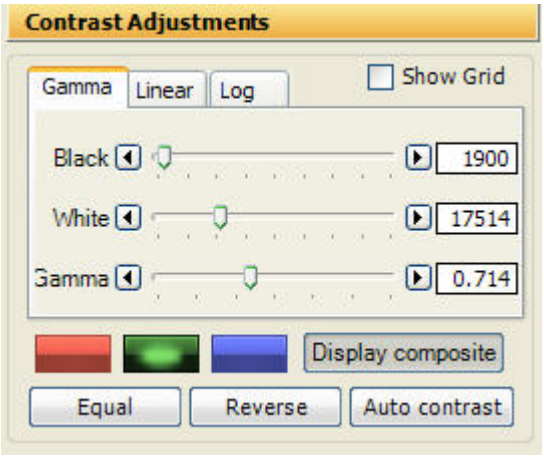


Figure 2.21 Contrast Adjustments window

The Red, Green and Blue buttons select the channel, and the Display Composite toggles the display between the color composite image and one of the single color channels. The three sliding scales adjust the black level, while level, and gamma.

Multi-channel (composite) selection

When a multichannel image is active the contrast adjustments window allows the displayed image as a color composite of the component channels. Multichannel images acquired with the auto contrast option selected will have the Black, White and Gamma levels optimized independently for each channel and each channel will have a different combination of settings. Multichannel images acquired with the auto contrast option unselected will have the Black, White and Gamma levels at the default values of 0, 65535 and 0.75 for each channel (respectively).

Use the **Contrast Adjustments** to optimize the display to enhance the features of interest in the image. Contrast adjustment tools modify the image display on the monitor only, and do not change the original quantitative data.

Adjust the scales by moving the slider, clicking on an increment arrow, or by typing a number in the value box followed by **Enter** on the keyboard.

To adjust the contrast of a three-color image, begin by selecting “Display Composite” (this is the default selection after acquiring a three-color image). Next, select the Red channel selector. Set the Black level to 0, and the White level to 65535. Gamma levels should be between 0.6 and 0.7. To reduce the normal background observed in the Red channel, slowly increase the black level. Once the Red background level appears visually Reduced, use the Gamma selector to increase the intensity of the Red bands. Repeat this protocol for the Green channel and Blue channel.

Single Channel Selection

When Display Composite is checked, the image display shows all three channels as a composite image. An single channel can be shown independent of the other color channels by selecting (clicking) the appropriate color channel then de-selection (clicking) of the display composite button. Moving the sliders will now change only the display settings of the actively selected color channel.

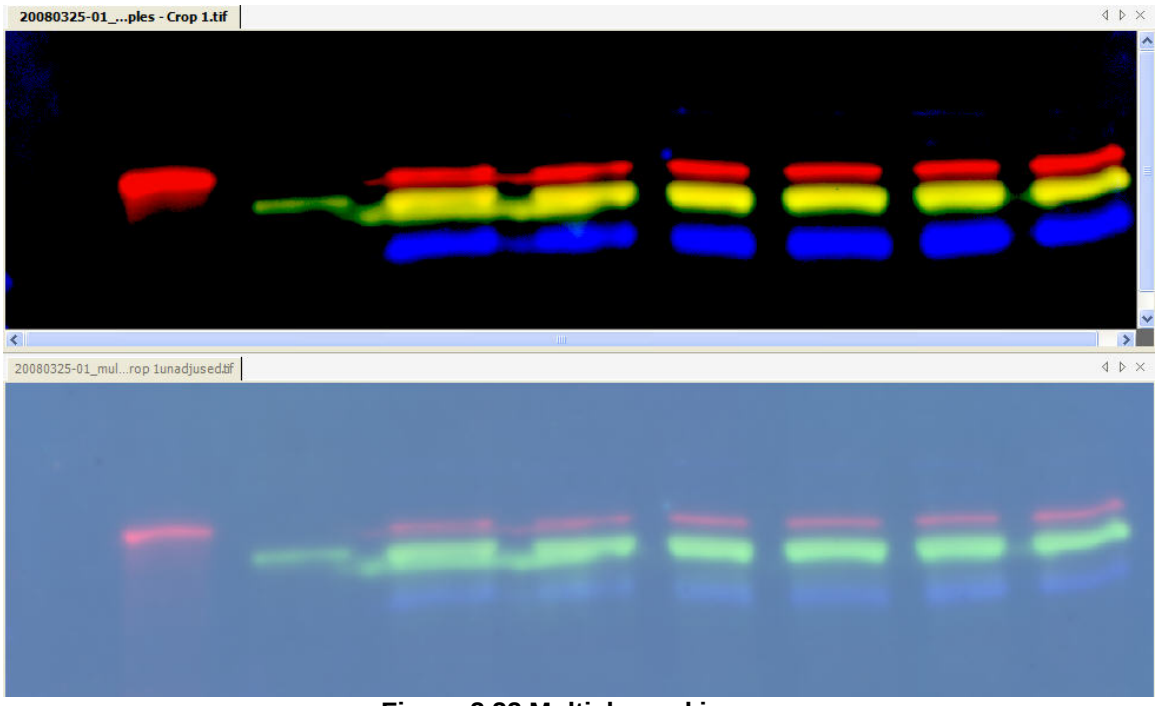


Figure 2.22 Multichannel image

Multichannel image with default contrast settings (bottom image) and with settings optimized for each channel (top image).

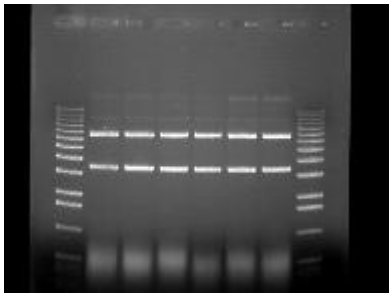
Contrast adjustments do not affect the raw data, but only change the visual appearance of the image. Performing data analysis on a contrast-adjusted image provides the same result as on a non-contrast adjusted image.

Automatic Enhancement

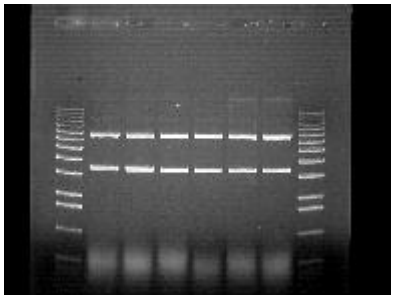
Figure 2.23 The Enhance Tools

This function is ideal for new or inexperienced users of the system since it offers 9 levels of automatic image enhancement of the black, white, and gamma levels simultaneously. For an inexperienced user, in can be difficult to adjust each black, white, and gamma buttons to their respective optimal positions. By clicking on one of the nine Auto Enhance Level buttons, the image is optimized according to a unique level. Button 1 will make the image 'darker'. Each increasing button click will 'lighten' up the image until button 9 is pressed which will make the image the 'lightest' possible.

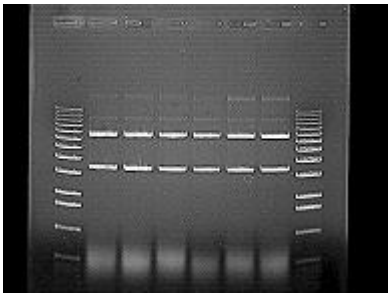
To undo any Auto Enhance Levels, just press on the Reset button on the main interface.



Original Image



Auto Enhance Level 2 Image



Auto Enhance Level 9 Image

Original Auto Enhance Toolbox

Auto Enhance Toolbox with level 2

Auto Enhance Toolbox with level 9

Tool Bar

The Tool Bar window provides intuitive icons for the most common functions in AlphaView.

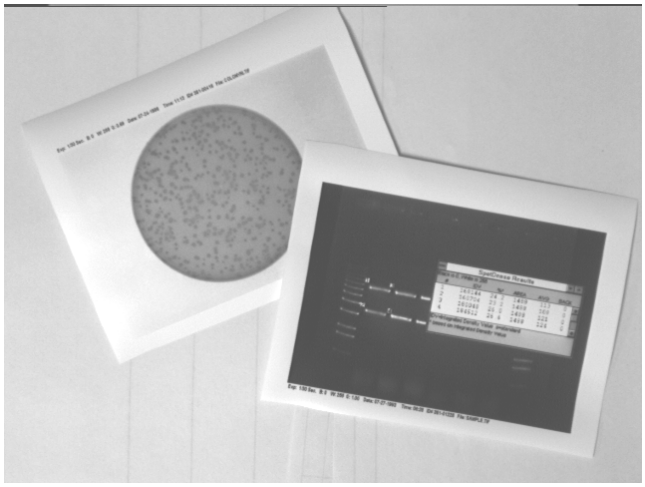
Figure 2.24 Tool Bar

The **Navigator** icon starts the “AlphaNavigator” Wizard.

The **Open** icon functions identically to the File Open function in the upper menu bar. This function is used to open previously saved images. Detailed instructions are available in Chapter 3.

The **Save** and **Save All** icons function identically to the File Save and File Save All functions in the upper menu bar. This function is used to save captured images to the desired storage medium.

Once an image is displayed, it can be printed on the default printer by clicking the **Print** icon in the Tool Bar window display. Most printers can be configured through the Windows operating system to be the default printer. Refer to your Windows operating manual for more information on installing a default printer.



Sample Printouts

The **Zoom Out** and **Zoom In** icons provide easy zooming ability while you are active in image enhancement or analysis functions providing increased versatility. Detailed instructions are available in Chapter 4 as this function is also available in the Tool Box**,** Enhancement Tools.

Note: The Status Bar always displays the image zoom setting in real time.

Assists the user to zoom in on a selected area, the average pixel is displayed..

The **Fit in Screen** adjusts zoom factor to fit image in available screen area.

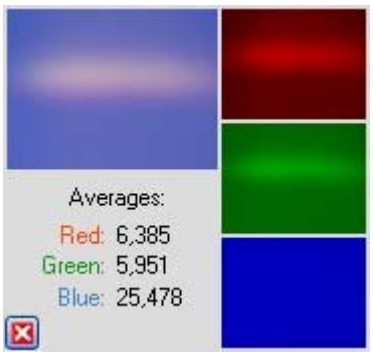
The **Image Drag** icon is useful for to pan with a zoomed image. To activate this function, click on the icon and move the mouse cursor to the image. The cursor will have changed to a small hand. Click the left mouse button and drag to move the image. When you are done, you can click the Image Drag icon again to deactivate it.
Note: Image Drag is only active when the image is zoomed in beyond 1X (greater than 100%). The icon is grayed out in other zoom modes.

The **Saturation** icon allows for a quick image display of saturation. Completely saturation black regions (gray scale 0) will turn green and saturated white regions (i.e. gray scale 255, 4095, 65,535) will turn red. This is a useful tool to check for linearity of an image before analysis occurs. Saturation is a feature that is most important during the acquisition stages and is thoroughly detailed in the acquisition features of the system manuals.

The **Image overlay** creates an RGB image from three grayscale images. Select the images for the Red, Green and Blue channels in the selection window

The **Extract Channels** icon is available when a Multichannel image is active. Extract channels produces a separate image for each channel.

The **Channel Viewer** icon is available when a Multichannel image is active.



The Channel Viewer opens a window that displays the multicolor image and each corresponding channel separately. The window may be moved around the image by simply dragging the window.
The average Red, Green and Blue intensities in the window are also displayed.

Clicking **Reset** returns the image to the system defaults as specified in the active default file. This is detailed later in Chapter 3.4 of the manual.

Clear removes any overlays currently displayed on the image. This function can be useful if annotations or other displays obscure parts of the image.

The **Notepad** icon opens up a dialog box to allow the user to quickly track experimental conditions, comments, and any other details to be saved as an electronic copy for future reference. Detailed instructions are available in Chapter 3 as this Notepad function is duplicated in the Utilities function in the upper header bar.

The **Open file explorer** opens windows file browser

The **Compare Image** tool allows comparing two or more images in compare view.

Tool Box

The Tool Box window contains an intuitive interface for performing all image enhancement and analysis functions.

Figure 2.25 Tool Box

The Enhancement Tools option contains the controls for enhancing and adjusting the image. This includes software filtering, false colors, zoom factors and other unique features. The Analysis Tools contain the controls for quantitative analysis including gel smiling corrections, band matching, Lane Profile densitometry, multiplex band analysis, molecular weight calculations, colony counting, and arrays. Both the Enhancement Tools and the Analysis Tools are detailed in chapters 4 and 5 of the manual respectively.

Status Bar

The Status Bar is located on the bottom of the monitor and provides a real time display of the mouse cursor x, y position, the image zoom factor, and the grayscale intensity at the mouse cursor x, y position.

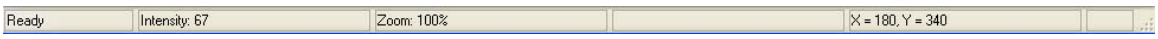


Figure 2.26 Status bar

Chapter 3. DROP-DOWN MENUS

Across the top of the screen is a Windows menu bar containing several system operation functions. These include file saving and loading, edit, image, setup, overlay, file utilities, view, window and help functions.

Figure 3.1 AlphaView Drop-Down Menu

The File Menu

Use this menu to save an image as a file, retrieve a previously saved image, select different printers, print an image to a parallel printer, overlay multiple images in RGB color channels, close an image, log-off of the system or exit the system.

Figure 3.2 File Pull Down Menu

File Open

This function opens an image, which has been previously saved as a TIF, GLP, BMP, PCX, TGA, PIC, JPG or Macintosh® TIFF (MAC) file.

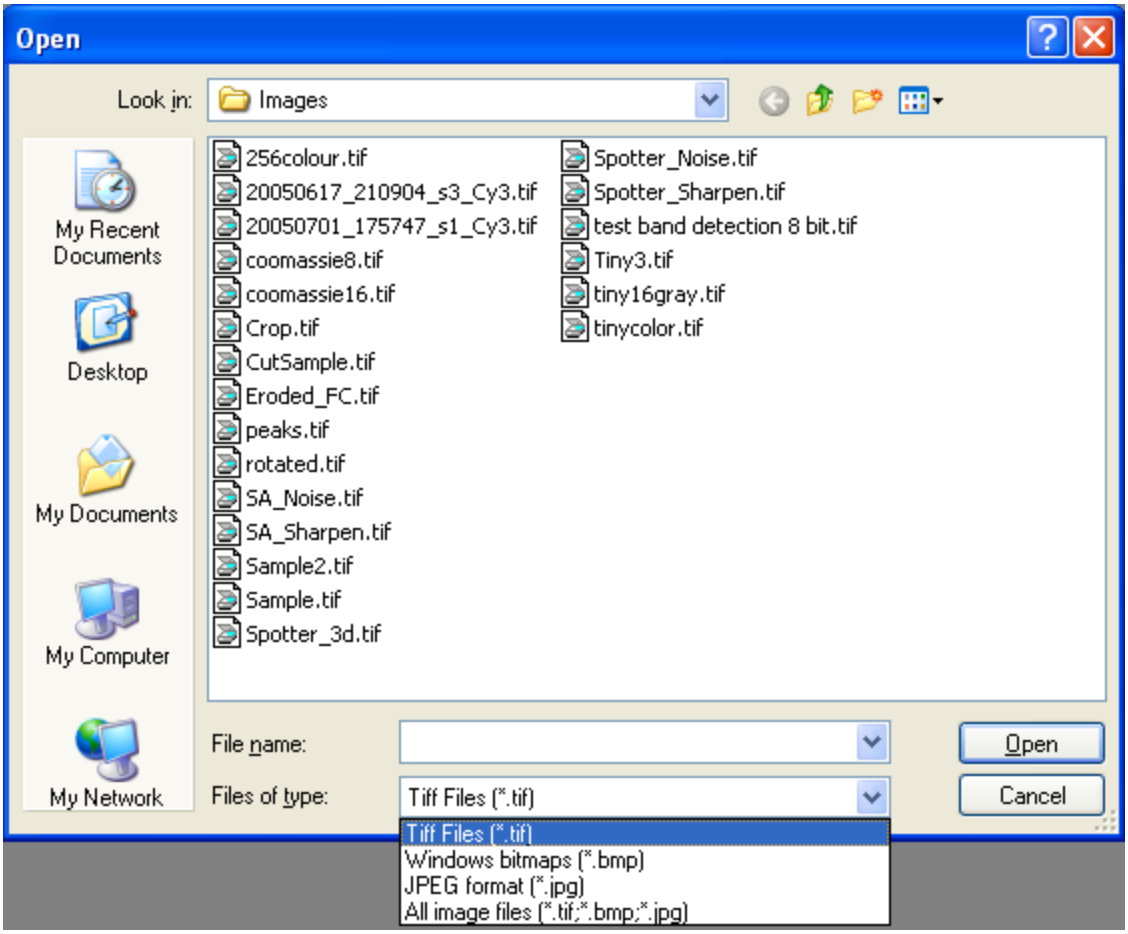


Figure 3.3 File Open Dialog Box

Using the left mouse button, click on the name of the file to be loaded. That name is then highlighted in the list and appears in the text box below the File Name prompt.

Alternate disk drives can be accessed using the “Look In” dialog box.

Once the file has been selected, click on the OPEN button to load the file. (Alternatively, double-click on the file name.) The dialog box disappears and the selected image appears in the image window on the screen.

To dismiss the dialog box without loading an image, click on the Cancel button.

Save/Load Analysis

You can use Save/Load analysis feature from file drop down menu, to save all your work and then load it back for later use.

Note: The load analysis menu item can only be accessed when the user opens any the analysis tools. If the image opened has an analysis saved, the item will be activated.

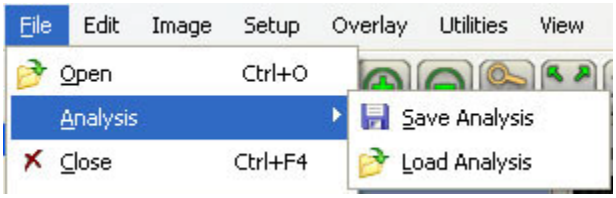


Figure 3.4 Save/Load analysis feature

File Close

This function closes the image currently displayed on the screen.

File Save, Save As, Save Modified and Save All

Save allows original images to be saved in several different formats. *Save As* allows images that have previously been saved to be saved in a different location or as a different file type without affecting the original image. *Save Modified* saves the image as a 8-bit color image with annotations burned into the image. *Save All* saves all images in .tiff format only.

AlphaView™ has the ability to save files in several formats, see the following figure:

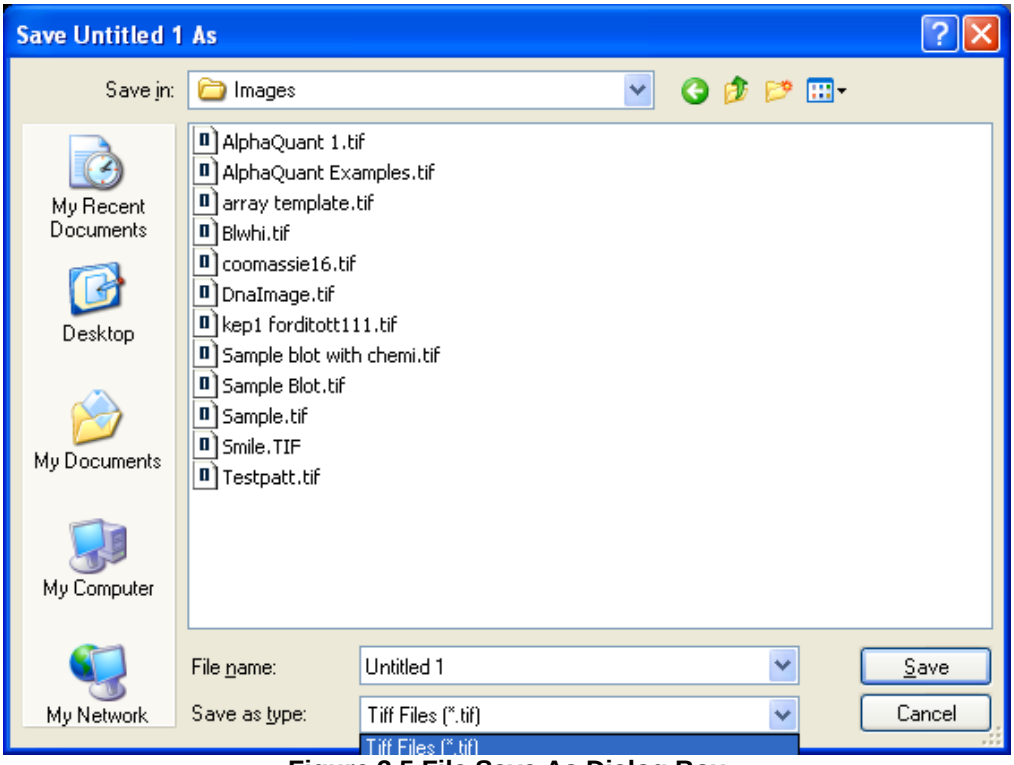


Figure 3.5 File Save As Dialog Box

Enter a new file name in the text box adjacent to the File Name prompt. Next, choose a file type from the Save as type list.

AlphaView™ will automatically give the appropriate 3-character extension. AlphaView™ will also create a file with the same base name and a .STP extension. This setup file saves information specific to this file, such as Black Level, White Level, Gamma Setting and 1D-Multi template placement. If the file is accessed later, these settings will be recalled.

File Types

TIFF is the default file format for AlphaView™ files. TIFF is an acronym for "tagged image file format" and was developed as a flexible and machine-independent graphic file format. Saving as a TIFF file will allow users to double-click TIFF files from Windows Explorer and automatically launch the application on any machine that has AlphaView™ loaded on it. Users may customize this in the preferences section covered in section 3.4 of the manual if they wish to change the default file type.

Mac TIFF is the Apple Macintosh® version of the TIFF file format. Mac TIFF files have the extension .MAC so they can be easily distinguished from Windows TIFF files. Most software can distinguish between Mac and Windows TIFF formats and can accept either. AlphaView™ offers the option of both formats in the event that only one of the two is acceptable.

GLP is a proprietary file format that allows changes to only be made in AlphaView™ programs. It will accept 8 bit and 16 bit images and can not be opened in any other software program.

BMP, PCX, TGA, PIC, JPG, GLP are additional graphic file formats which may be useful when saving an image for desktop publishing. These file formats can be imported directly into many Macintosh® and PC programs. (See Appendix A for more information.) Do not use these formats to save images that will be analyzed later, since pixel data can be lost or altered when saving files in these formats.

Note: Not all of the file types listed above can be saved as a 16 bit file. The AlphaView software allows 8 bit Tiff images to be saved as BMP, JPG, or Tiff format. 16 bit Tiff images can only be saved as Tiff images. Some may require you to convert the image to an 8 bit file first.

Original versus Modified Files

An Original image file is one in which the data is saved in an unaltered form. This option should be selected if the image will be analyzed later. If the Black level, White level, or Gamma settings have been adjusted, the new values are saved but the *pixel values are not altered*. When this file is opened at a later time, AlphaView™ will display it with the values that were displayed when the image was saved, however, it is still possible to revert to the original raw image file by selecting Reset on the Tool Bar.

Annotation information cannot be saved with the Original image option. (It can, however, be saved as an Overlay. See section 3.5 for more information.)

If the image was saved as an original file using an older Alpha Innotech system, some distortion may occur when viewing it in desktop publishing or word processing programs. If this occurs, save a copy of the image in the Modified format before importing it into another software package.

An image that is saved as a Modified file permanently retains the changes to the image's Black level, White level, and Gamma setting. Annotations and any filtering performed are also saved with the image, replacing original image information with the new information.

Note: If the image is saved as a Modified file it is converted to an 8-bit image.

Print

This function sends the image to the default printer specified in Print Setup.

Print Setup

This function displays a dialog box in which the settings for the parallel printer are specified. When all the pertinent printing preferences have been specified, click on the OK button. If you purchased a printer with AlphaView, this will be preset from the factory.

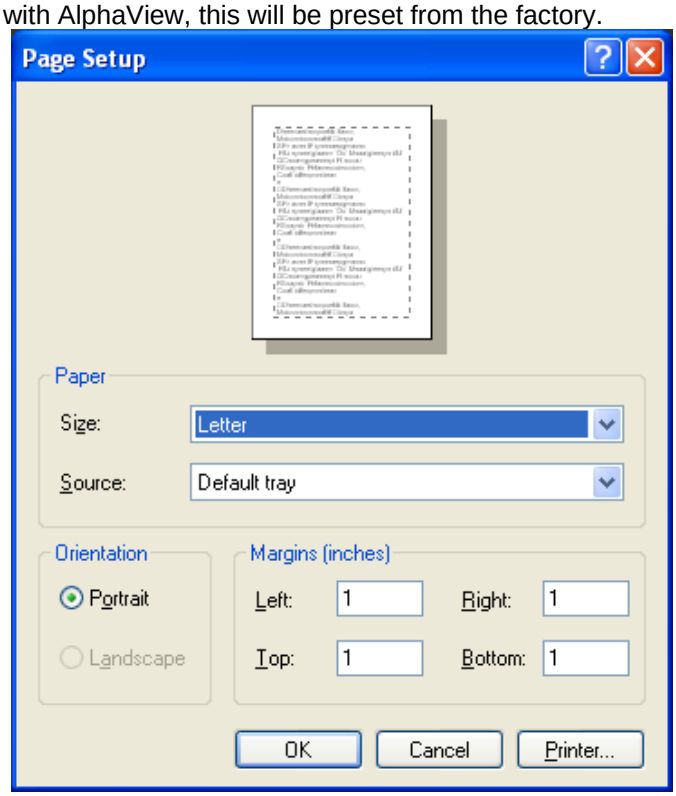


Figure 3.6 Printer Setup Dialog Box

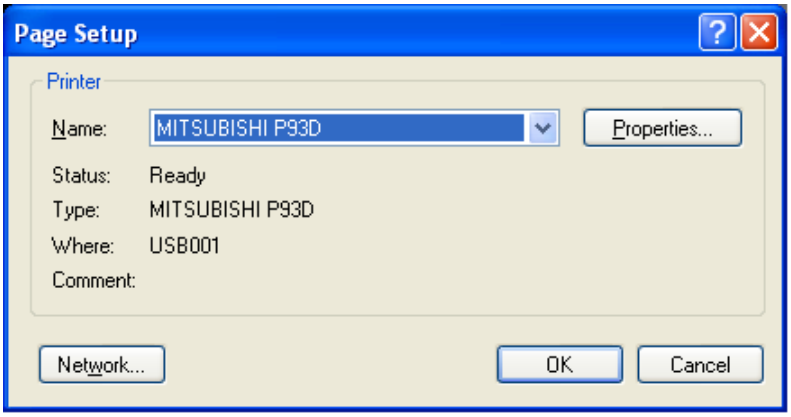


Figure 3.7 Printer…. Dialog Box

For more information on using the Print menu, see the Windows manual.

The Exit Function

The Exit function closes AlphaView™. To restart AlphaView™ from Windows, double-click on the AlphaView™ icon.

The Edit Menu

The Edit menu provides the ability to copy, crop and remove any annotations or filters that have been added to the original image.

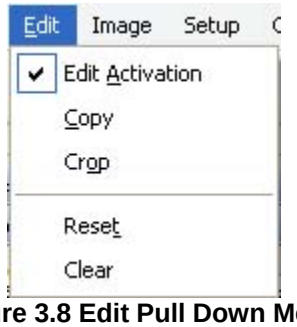


Figure 3.8 Edit Pull Down Menu

To activate the Copy and Crop functionality, place a check mark next to **EDIT ACTIVATION.** This will turn the mouse cursor into a + sign that will allow you to highlight the region of interest for the image. After Edit Activation is highlighted, the desired area of interest is drawn using the mouse.

Figure 3.9 Ready to Crop or Copy

Once this is completed, you can select either the **COPY** or **CROP** function in the EDIT menu options.
COPY will copy the desired area of interest into the Windows Clipboard and allow you to paste into any desktop publishing package (i.e. Word, Excel, Adobe Photoshop, etc.).
CROP will display just the region of interest as the active window in the AlphaView interface.

Figure 3.10 AlphaView interface after CROP has been selected

Reset and Clear

The Reset option configures the Black, White, and Gamma settings to default settings. Clear removes any annotations that are present on the image.

The Image Menu

The Image menu option provides the ability to perform a variety of image processing functions.

Figure 3.11 Image Pull Down Menu

Overlay

To superimpose images, use the OVERLAY function under the Image File menu. This function will display separate multiplexed images or a RGB color image as a compiled image with the appropriate color channel images added together. A simple way to acquire multiple images for this function is to use the Movie Mode function in image acquisition and acquire a series of identical images.

The Overlay Images option allows you to overlay up to three different images with three different color channels. You can select the BROWSE button for each color channel and select the appropriate images to be used for generating a color image. For example, if you have a saved grayscale images of an identical gel taken with a SYPRO red filter for the red stain and a SYBR green filter for the green stain, you can choose these images in the appropriate Red and Green Channels to generate a composite image with the red and green colors mapped onto the compiled image.

Note: The images must be the same bit depth and resolution for the software to overlay the images.

Extract Channels

Extract channels produces a separate image for each channel from a Multichannel image and display them on image screen window.

The Extract Channels icon is available when a Multichannel image is active.

Channel Viewer

The Channel Viewer icon is available when a Multichannel image is active.

The Channel Viewer opens a window that displays the multicolor image and each corresponding channel separately. The window may be moved around the image by simply dragging the window. The average Red, Green and Blue intensities in the window are also displayed.

The Channel Viewer can seamlessly be used in Comparer View by moving Channel Viewer over any image.

Equalize

The equalize option performs a duplicate function to the EQUAL option in the Contrast Adjustment Window. This is a useful function for detecting faint bands on a sample.

Arithmetic

The Arithmetic function is used to add, subtract, average and divide several images together to generate a compiled image.

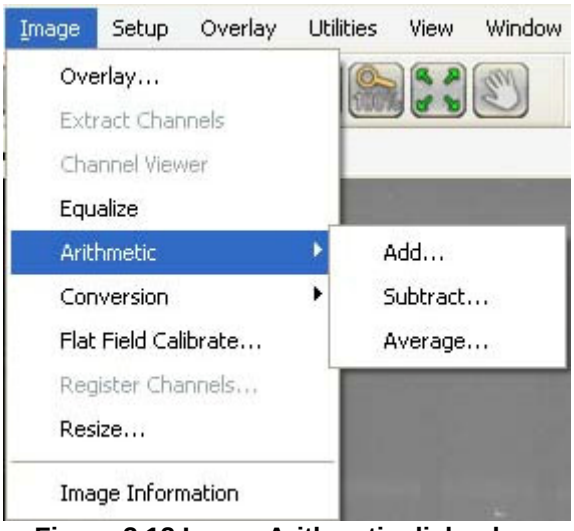


Figure 3.12 Image Arithmetic dialog box

To average a set of images together open one of the images in the set and then select 'Average a Set…' under the Image pull down menu. A prompt will appear allowing the user to select all of the images that for the set. It is possible to browse the directories looking on the network drives and removable media if necessary. Once all of the images have been selected click on the open button to finish the image set. The resulting image is an average of all of the images together. This is a useful function for extending the dynamic range on a set of similar images by allowing bright spots and faint spots to be seen on the same image.

The other functions are adding, subtracting and dividing images together. Adding together images is frequently used for colorimetric markers run together with chemiluminescent samples. Subtracting images is often used to remove noise from a sample by running dark images first and subtracting them out of the final image. The most common application for quotient is for those technical users who run their own flat field corrections. This can be done using the Flat Field Calibrate selection under the Image pull down menu which will be described in detail later in this section.

All three of these arithmetic functions are performed by opening the main image that will be adjusted. Next select the appropriate arithmetic function under the image pull down menu. Then select the image that is to be added, subtracted or divided from the original image and select open. The dialog box will disappear and the resultant image will appear.

Note: Images that have been arithmetically altered are ideal for publications and documentation, however, they are strongly not recommended for analysis as the pixel values have been adjusted.

Conversion

Since AlphaView™ can generate 16-bit files, the conversion option is useful when an image is to be imported into a program that only accepts 8-bit images. Choosing this option will convert a 16-bit image into an 8-bit image.

A 48bit color image can be converted into a 16 bit greyscale image by simply averaging the RGB values for each pixel.

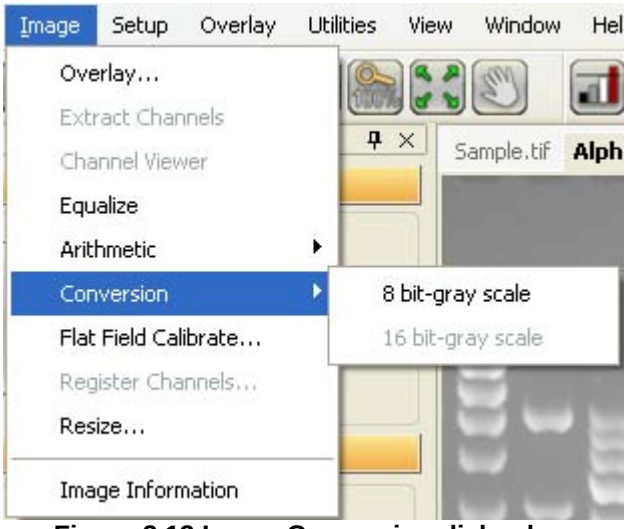


Figure 3.13 Image Conversion dialog box

Flat Field Calibrate (Manual)

Flat Field Calibrate is a function that is used to 'flatten' the image so that the pixel data is even across the entire image area. This is a function that is useful for large gels and other applications that use the entire field of view for an image. Flat field calibration is already performed automatically as part of the acquisition process – perform manual flat field correction only to images that have not already been flat field corrected. Use the image info function (see Figure 3.15) to identify the image processing operations performed (look under Post Corrections Entry) on the image in question before proceeding.

Creating flats can be art in itself; there are many documents on the internet that can help users interested in this arena to create the ideal flat for the application. However, some useful flats that have been created in the past involve very simple tools like a piece of 8.5 x 11 regular low quality copy paper (higher quality paper contains watermarks that will show up in the final image). It is essential that both the flat and the gel images be identical, including the aperture, zoom (if applicable) and focus settings on the lens.

Step-by-Step Manual Flat Field Calibration Applied to existing image:

a) Place the gel or other application in the cabinet or dark room.
b) Adjust the aperture, zoom (if applicable) and focus on the lens.
c) Use auto-expose set to the normal selection and acquire an image of the gel. (Alternatively, it is possible to select show saturation and then use expose preview and adjust the exposure time manually to just under saturation.)
d) Save the image of the gel.
e) Next remove the gel from the UV transilluminator or white light tray and clean and/or dry off the surface if necessary using glass cleaner.
f) Place the white piece of paper onto the appropriate surface. (For example, if the UV transilluminator was used, place the paper onto the UV transilluminator; if the white light tray was used, place the piece of paper onto the white light tray.)
g) Turn on the appropriate light source used (white light, UV transilluminator, epi lights, etc.).
h) Without changing anything on the lens acquire another image of the 'Flat' image following step #3 again.
i) Save the Flat image.
j) Open the original gel or other application image.
k) Select Flat Field Calibrate from the Image pull down menu.
l) Browse the directories for the 'Flat' image created.
m) Click on open. Make sure to save the flat field calibrated image for future use.

Register Channels

When bands of different channels are not aligned, multichannel Images may be registered (aligned) using the Channel Registration tool.

1. Select Register Channels from the Image menu.
2. Place two ROIs in the image according the instructions.
3. Click on the Register Channels button.

(Image translations are calibrated and applied as part of the acquisition process for multichannel images -not for gray images).

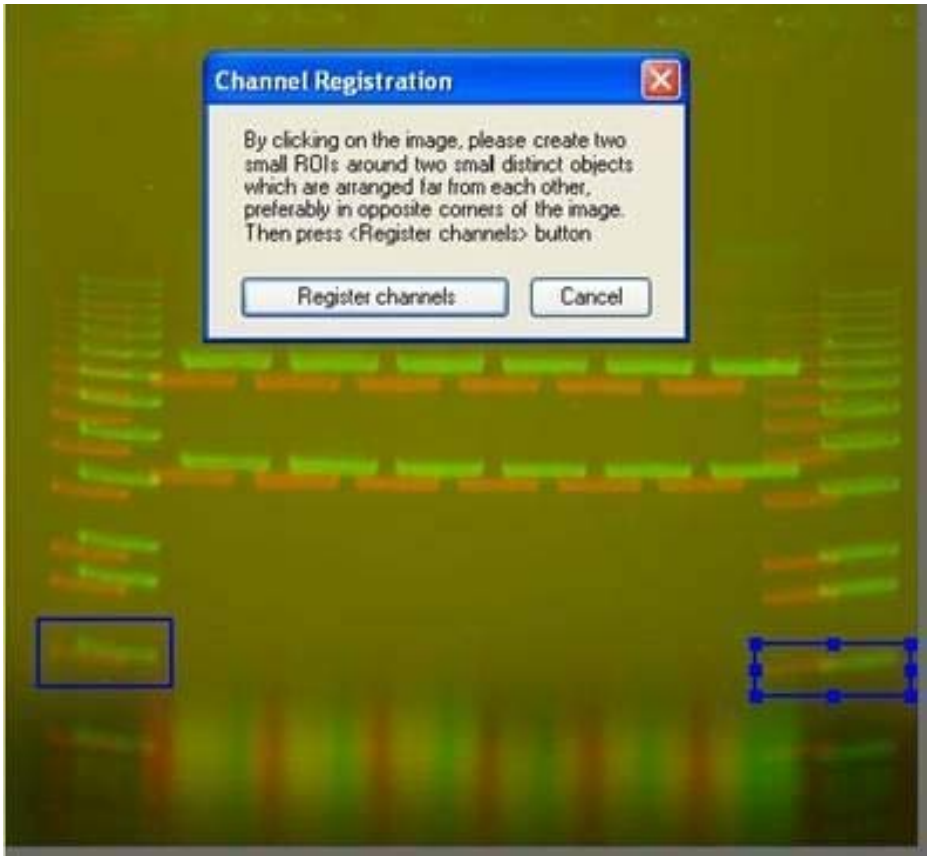


Image Resize

The image resize function is to resize an image to a specific dimension for use in graphical presentations. You have the option to 'Preserve aspect ratio' to avoid image dimensional distortion, or you can deactivate this function and configure the image resolution to the desired Width and Height dimensions.

Figure 3.14 Image Resize dialog box

Note: It is recommended that you DO NOT perform quantitative analysis on resized images.

Image Info

The Image Info function provides a dialog box with all detailed image properties. To remove this dialog box from the screen, click on the OK button.

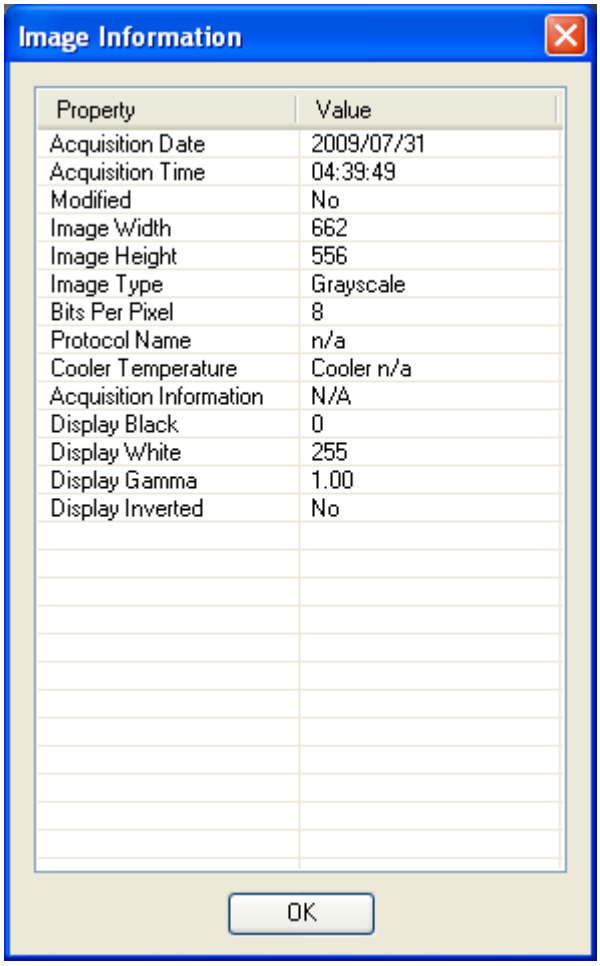


Figure 3.15 Image Info dialog box

The Setup Menu

This menu customizes the system settings by allowing users to save default parameter preferences and customize the software settings.

Figure 3.16 Setup Pull Down Menu

Print Info

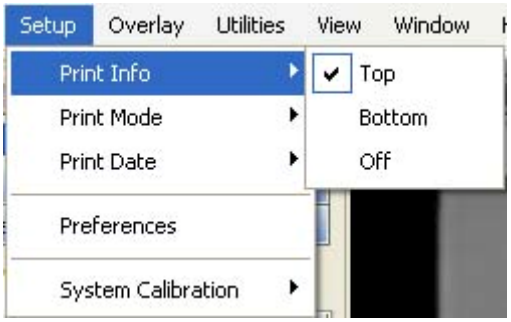


Figure 3.17 Setup Print Image Info Dialog Box

When printing an image, basic image information is included on the print. This includes the exposure time, the Black level, White level, and Gamma setting, the date and time the image file was generated, an image ID number, and the name of the file to which the image is stored.

To print this information at the top of the print, choose Top from this menu. To print at the bottom of the print, choose Bottom.

Note: Printing image information at the top or bottom of a print may obscure a small portion of the image. To print the image with no information on it, choose Off.

Print Mode

AlphaView software provides custom printing options. Printing can be achieved in three different methods.

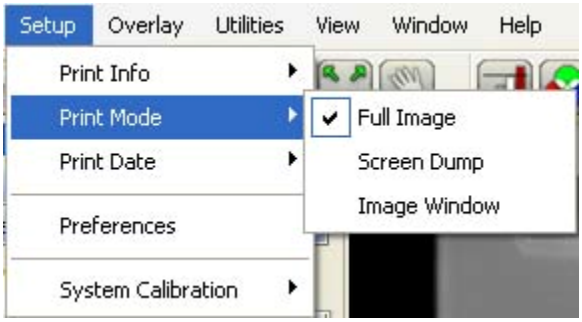


Figure 3.18 Setup Print Image Info Dialog Box

Full Image: Prints the original image. Does not print zoomed images or images overlaid with data screens.

Screen Dump: Prints the imaging area. Well suited for printing images overlaid with data screens and/or graphs, zoomed images, etc.

Image Window: Prints the highlighted window.

Print Date

Under the "Setup" menu there is a selection labeled "Print Info". This allows the user to change the format in which the date is printed. The choices are MM/DD/YYYY and DD/MM/YYYY.

Preferences

In order to change the preferences of the system, you will need to find the administrator of the AlphaView software program to log in –the login is enforced only on data altering tool tabs (image acquire, cabinet settings, and auto enhancement). If you do not have an administrator of the AlphaView software program, see Appendix C in this manual.

Figure 3.19 Login Dialog box for Preferences

There are five tabs in the Preferences menu (AlphaView Stand Alone software does not contain the Image Acquire or Cabinet Settings tabs):

1. General – Configure prompts and file saving/opening formats.

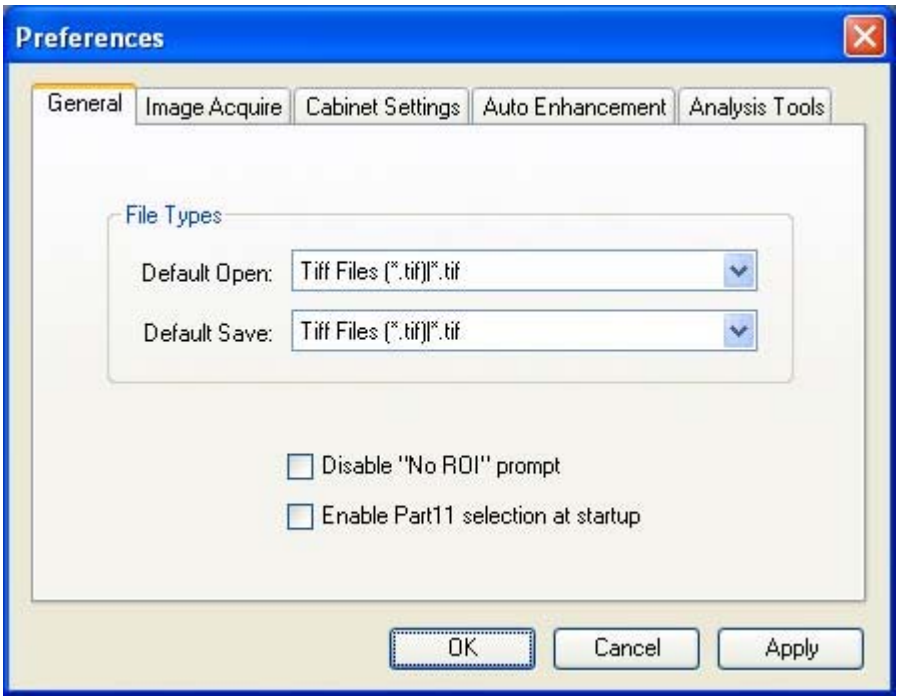


Figure 3.20 Preferences - General Tab

2. Image Acquire – Inverts the image seen by the camera and adjusts the ROI values

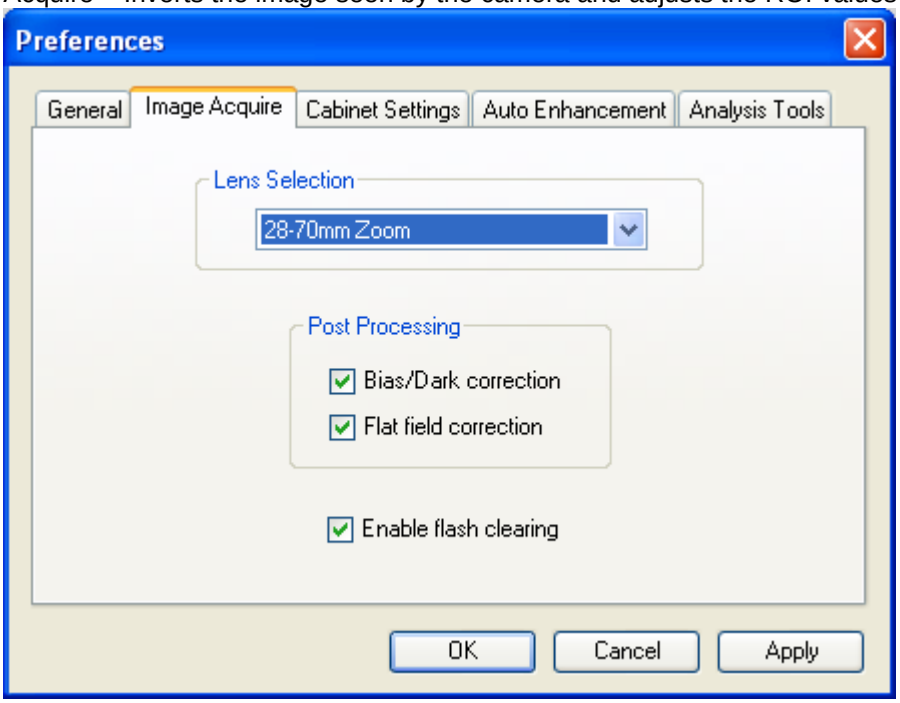


Figure 3.21 Preferences - Image Acquire Tab

Caution: We do not recommend that you make any changes to these settings without contacting technical support first. Some features on this tab are not currently implemented in the software.

3. Cabinet Settings – Used for adjusting the port settings and customizing filter positions on the cabinet.

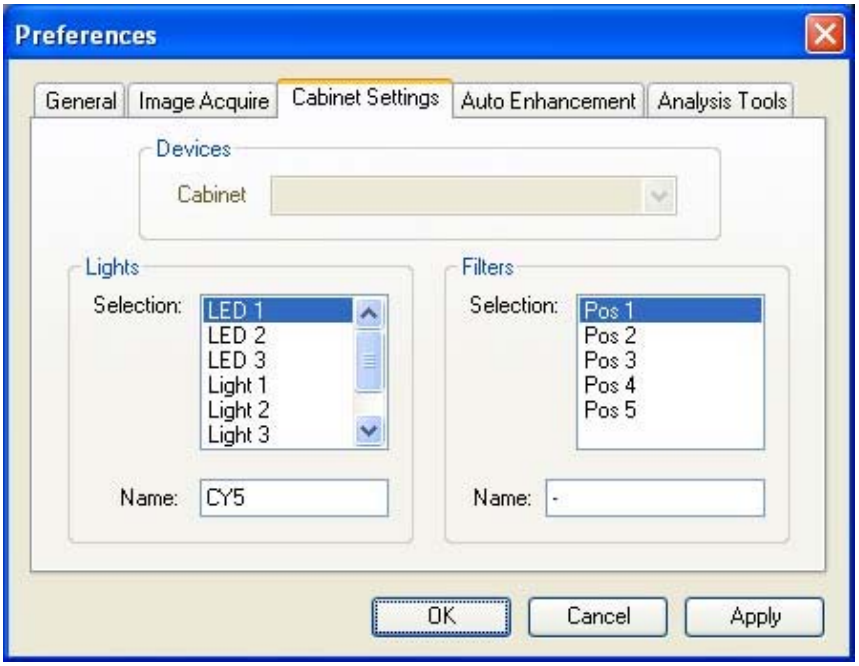


Figure 3.22 Preferences - Cabinet Settings Tab

4. Auto Enhancements – Used to customize the Auto Enhance Levels located in the Image Enhancement tool box.

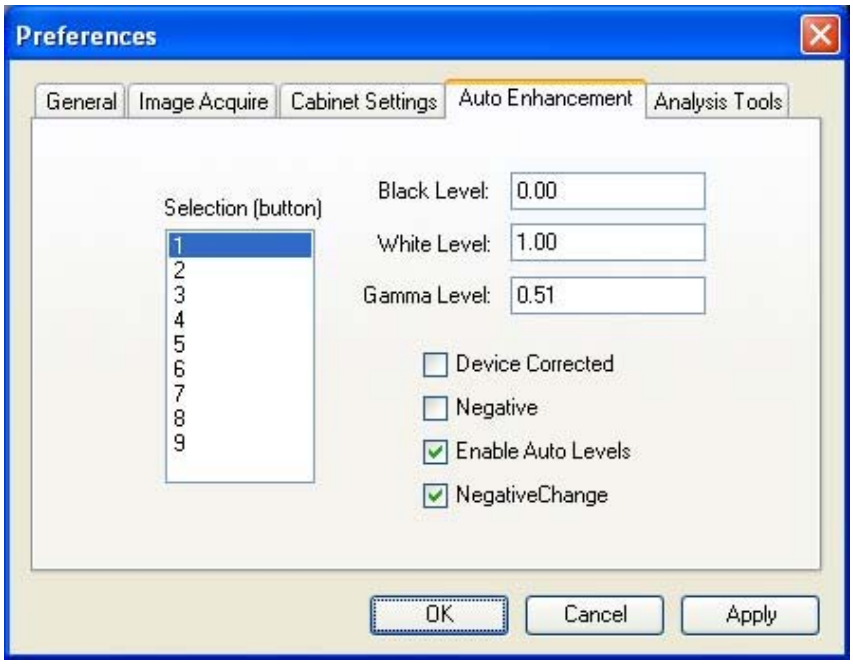


Figure 3.23 Preferences - Auto Enhancement Tab

To make changes to the preferences, select your change by typing in a new value or select/de-select the appropriate box with a check mark and then select apply. Some settings may require that the software be re-started for the change to take effect.

5. Analysis Tools – used to show/hide additional image analysis tools. Any change in the settings will require software be re-started for the change to take effect.

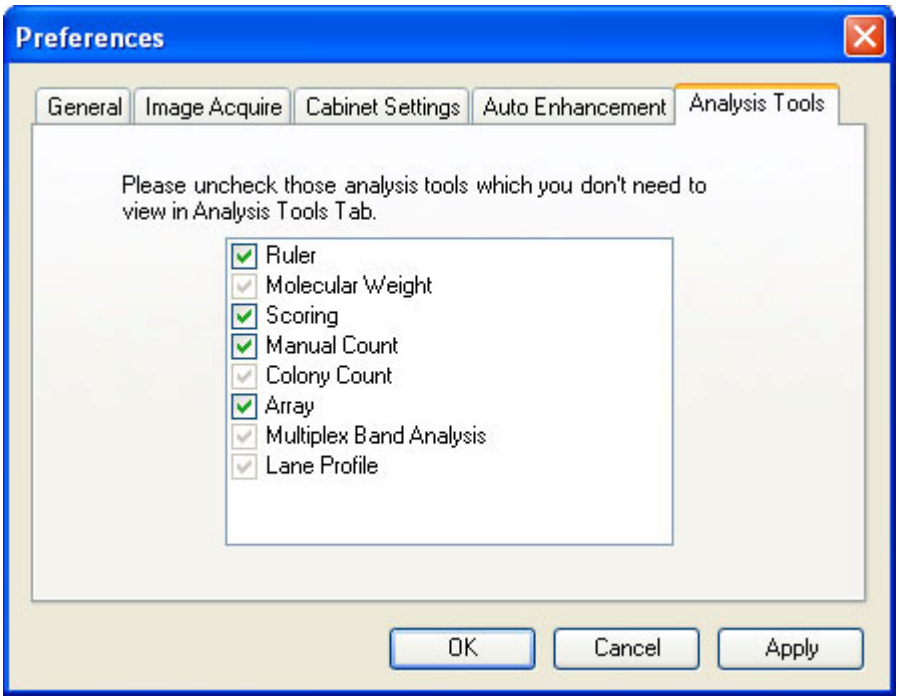


Figure 3.24 Preferences – Analysis Tools Tab

System Calibration

In order to use system calibration, it will be required the administrator log in of the AlphaView software program.

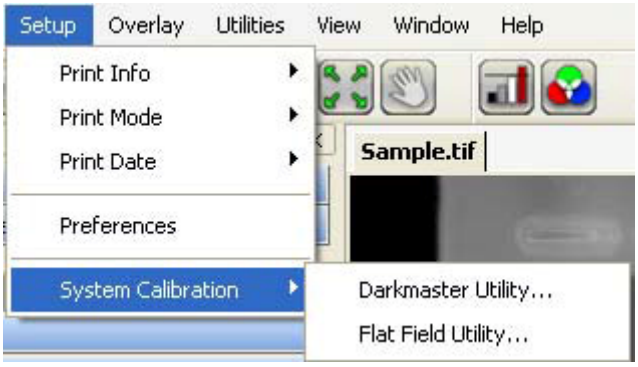


Figure 3.25 Setup – System Calibration menu

Darkmaster utility – see appendix D

Flat Field Calibration (Auto) – see appendix E

The Overlay Menu

The Overlay menu provides a means of saving and retrieving annotation overlays. This is especially useful when a standard gel format is run repeatedly. Lane numbers, molecular weight marker sizes, and other pertinent information can be stored as an Overlay file and retrieved at a later date. This eliminates the need to re-enter the information each time a new image is captured.

Figure 3.26 Overlay pull down menu

An overlay is any set of annotations (text, boxes, arrows, etc.) that have been drawn on the image. They can be saved as a group and opened later. If repetitive samples are being imaged, an overlay eliminates the need to re-enter the same information (such as lane numbers, standard sizes, etc.) continually.

Loading an Overlay

The Load Overlay function allows Overlay files to be retrieved and applied to the image currently displayed.

Opening an Overlay after an image has been captured places the annotations on top of the image. They can be stored as part of the image by saving the file as a modified file. (See Save Image As in Section 3.1 for instructions.)

Select the name of the file to be loaded. (If necessary, change the directory or drive.) The file name is then highlighted in the list and appears in the text box below the Filename prompt.

Once the file has been selected, click on the OK button to load the file. (Alternatively, double-click on the file name.) The dialog box disappears and the annotations in the selected file appear on the image.

To dismiss the dialog box without loading annotations, click on the CANCEL button.

Saving an Overlay

Once annotations have been made, select Save Overlay from the Overlay menu.

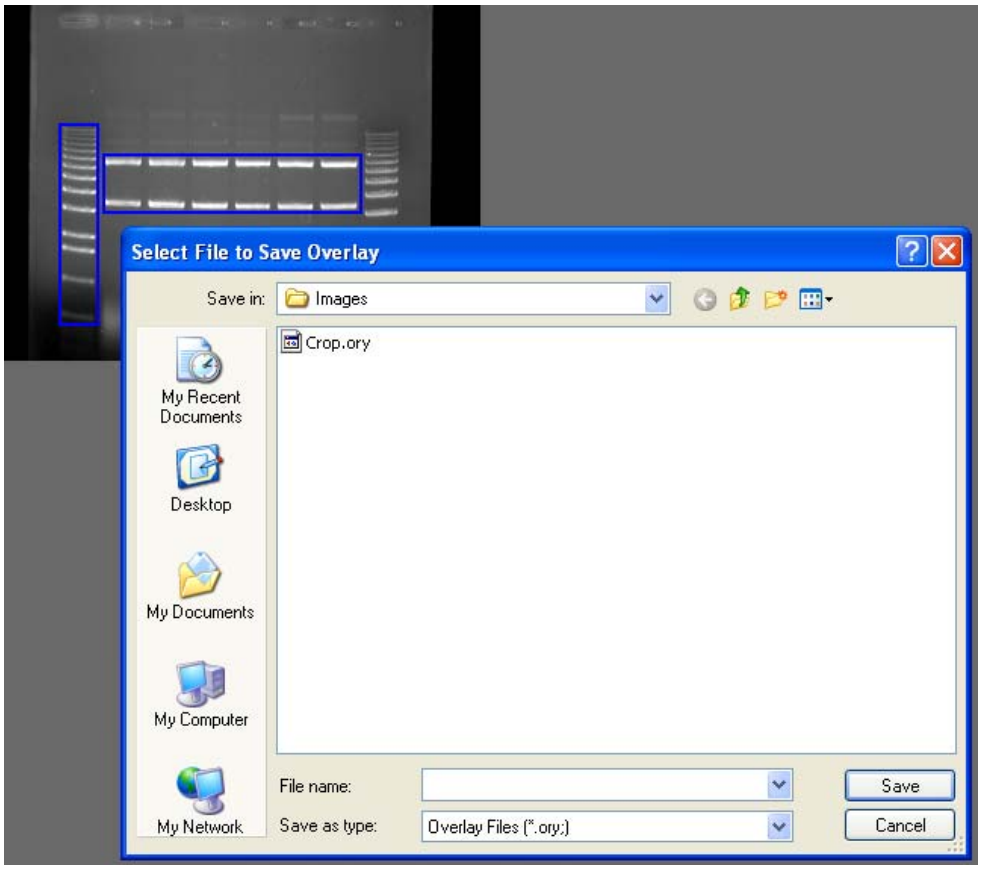


Figure 3.27 Save Overlay Dialog Box

Enter a new file name in the text box below the File Name prompt. AlphaView will automatically give the appropriate 3-character extension.

The current directory is the one in which the new overlay file will be saved. If necessary, change the directory or drive as described in Section 3.1.

Once a name has been entered and the appropriate directory has been accessed, click the SAVE button to save the overlay file.

Overlay Libraries

AlphaView contains a library of overlays that can be accessed through the Load Overlay function described above. This library of overlays is stored in the Image folder located in the AlphaView directory:

08WHITE.OVR / 08BLACK.OVR	8 lane labels in white/black
10WHITE.OVR / 10BLACK.OVR	10 lane labels in white/black
12WHITE.OVR / 12BLACK.OVR	12 lane labels in white/black
15WHITE.OVR / 15BLACK.OVR	15 lane labels in white/black
24WHITE.OVR / 24BLACK.OVR	24 lane labels in white/black
HINDIII.OVR	λ*Hin*dIII label

The objects in these overlays can be repositioned, resized, re-colored, copied or deleted as needed.

Show Annotation

To displays or hides annotations in Analysis modules.

The Utilities Menu

A number of functions are now handled by Windows programs. To access many of these programs while in AlphaView, open the Utilities menu and select the program of choice.

Figure 3.28 Utilities pull down menu

Notepad

The Notepad is a blank screen that allows the user to make notes about the experiment and save them as an ASCII file. The Notepad is useful for saving any imaging comments or experimental conditions with the saved image for future reference.

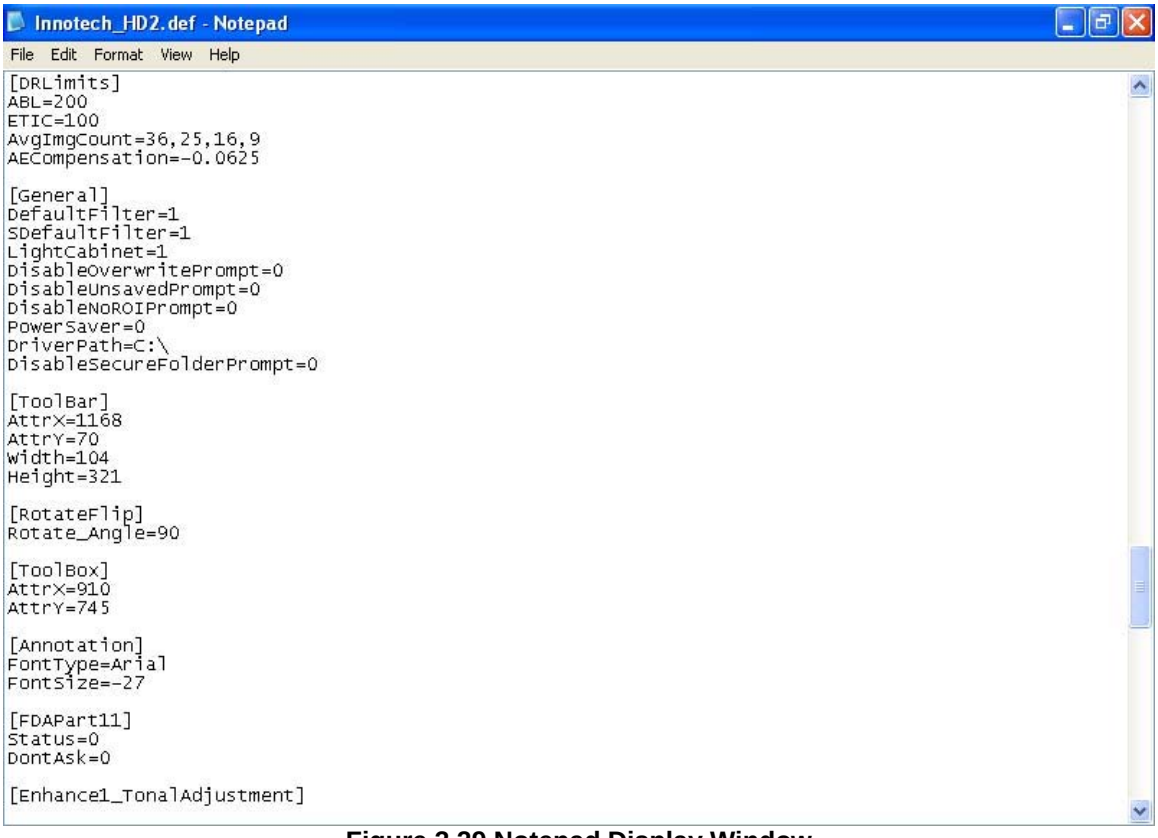


Figure 3.29 Notepad Display Window

Explorer

Windows Explorer allows access to files and other information saved on the local machine or the network, if applicable.

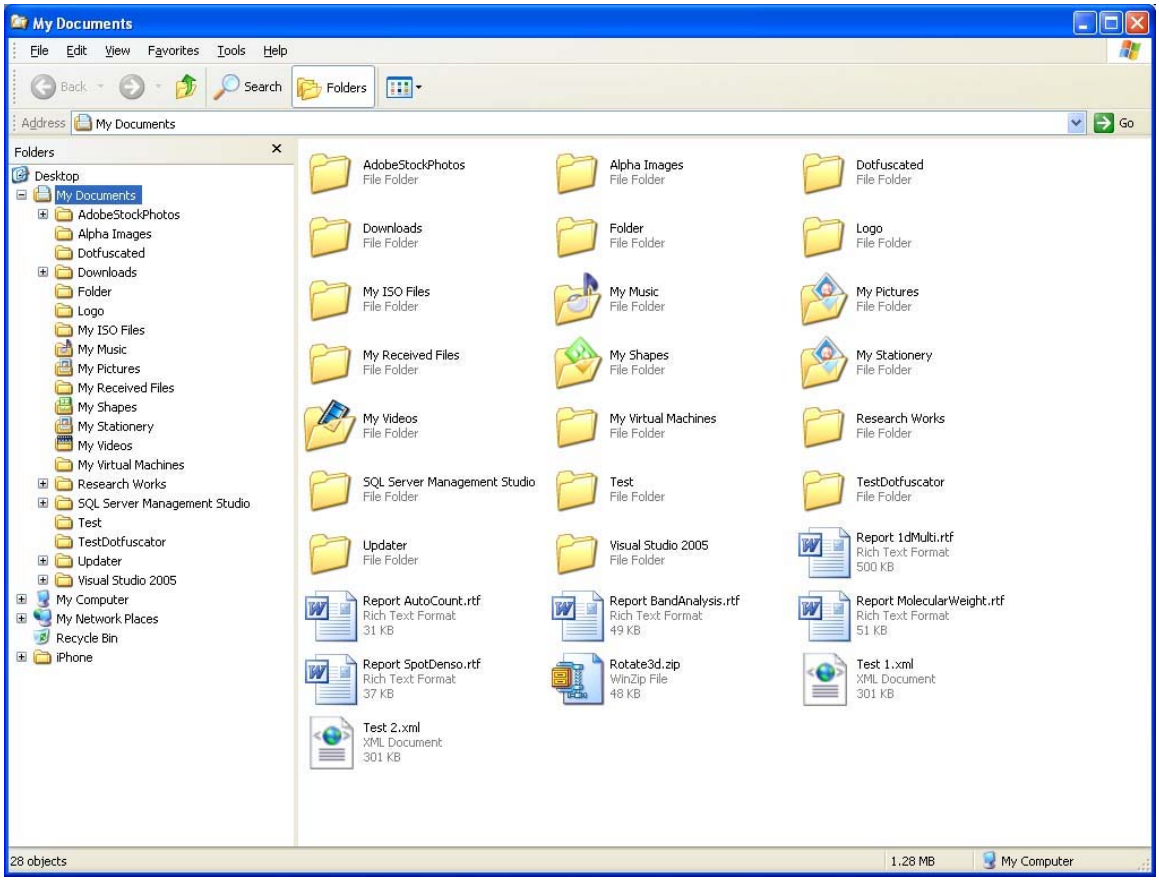


Figure 3.30 Windows Explorer Dialog Box

The View Menu

The View function provides the ability to control the display of the on-screen control tools as well as provide image enhancement abilities.

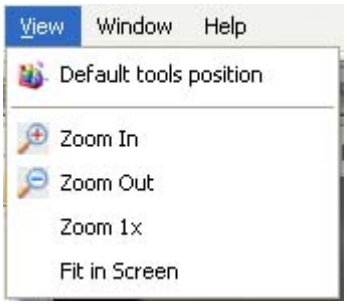
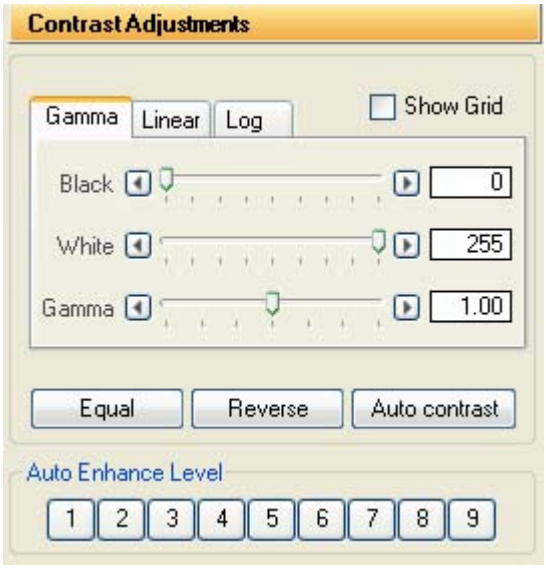


Figure 3.31 View pull down menu

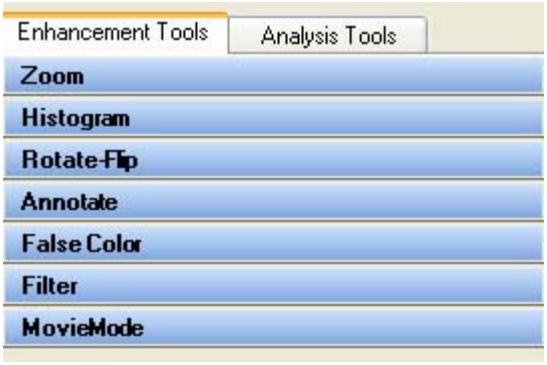
Default Tools Position

Four (4) main control windows exist within AlphaView: Tool Bar, Contrast Adjustment, Tool Box, and Status Bar:

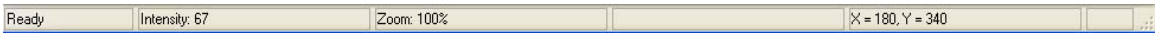
Tool Bar



Contrast Adjustment Window



ToolBox Window



Status Bar

These control windows automatically open when AlphaView is launched for additional ease of use and to generate a common 'look and feel'. Since these items are 'floating' tools, you can click on **Default Tools Position** to move all tools to the default locations for more intuitive operation. Lastly, except for the status bar, it is possible to select and move any of the other windows to a custom location.

Zoom Functions

Additional options provide the ability to **Zoom In** and **Zoom Out** on the image**, Zoom to 1X** and to **Fit to Screen**.

Note: Zoom In and Zoom Out are duplicate functions for the Zoom In and Zoom Out icons in the ToolBar and the Zoom options in the Enhancement Tools.

The Window Menu

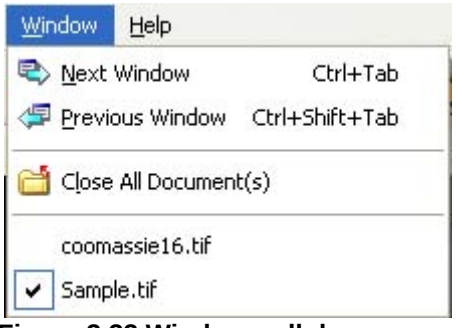


Figure 3.32 Window pull down menu

The Window menu function provides the ability to navigate from one image window to next image window. It also allows user to close all document,

The Help Menu

Figure 3.33 Help pull down menu

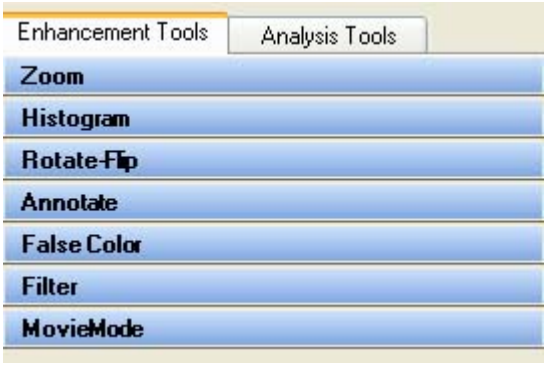
How to use this software help is available in the **On Line Help** section of the Help menu. Common tips are included for both Enhancement Tools and Analysis Tools detailed in Chapters 3 and 4 respectively.
The software must be registered before the end of the trial period. Registration can be done when the application is run or from this menu. **Note:** The registration menu item will be grayed out after successful product registration
To display system information, select the About option in the Help menu. This button accesses a pop-up box. This box shows the system serial number and software version number. Use this information when contacting Alpha Innotech for technical support, software upgrades, etc.

Figure 3.34 AlphaView About Help Dialog Box

To close the box, click on the OK button.

Chapter 4. THE IMAGE ENHANCEMENT TOOLS

Image enhancement tools are contained within the **Tool Box** as indicated. This tool set allows the user to zoom the image, rotate-flip the image, show the image histogram, perform automatic image enhancement, annotate on the image, display false colors, apply software filters, and activate the Movie function. Many image enhancement tools do not function with multichannel images.



The Zoom Tool

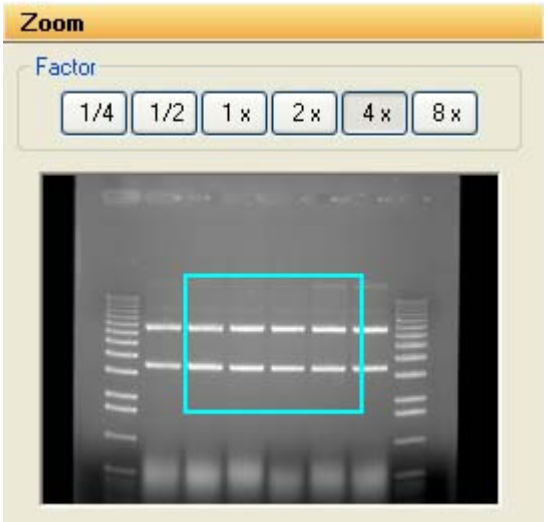


Figure 4.1 The Zoom Tools

The Zoom tool is found in Tool Box, Enhancement Tools

This function magnifies an image, making details easier to see, and allows movement around the magnified image.

An image can be displayed ¼x, ½s, 1x, 2x, 4x, or 8x larger than the original display by clicking on the appropriate buttons. To return to the original magnification, click on the 1X button.

When an image is magnified, only part of it can be displayed on the screen at any one time. To see different parts of the magnified image, use the green Pan Control box. The outer box shows a thumbnail of the entire image while the inner green box represents the portion currently displayed on the screen.

To view different regions of a magnified image, move the cursor into the inner box. Click and hold down the left mouse button. The cursor changes to a hand; use it to drag the green box until the desired region of the image appears on the screen.

Alternatively, use the scroll bars in the image window to move the image up/down, and left/right.

On-screen Zoom tools is also available located on the main ToolBar Window in. This function duplicates the Zoom tool in Tool Box, and also allows for Image Drag to easily pan the image during any analysis functions located in Tool Box Analysis Tools.

Image Drag icon in ToolBar

Zoom Icons in ToolBar

Histogram

The histogram is a graphical display of the proportion of pixels assigned to each of the 4,095 gray levels. This tool is found in Tool Box, Enhancement Tools.

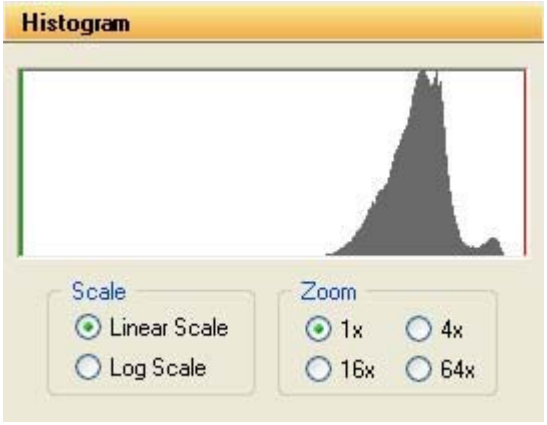
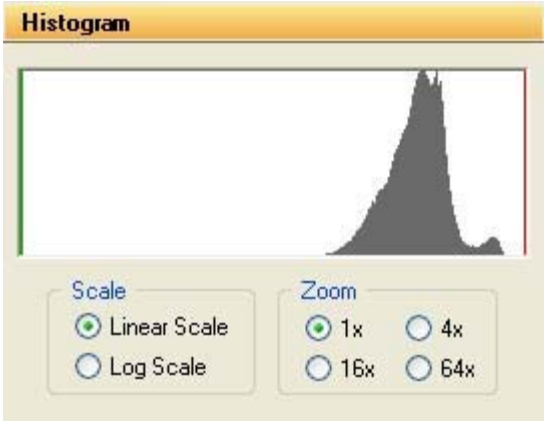


Figure 4.2 Histogram display in the Tool Box

The image is made up of picture elements (pixels) having brightness levels ranging from black to white. A very bright image will have most of its pixels registering high gray levels and conversely, a very dark image will have most pixels with low gray levels (approaching zero).

The histogram is displayed in the lower left corner of the screen, below the image window. The horizontal axis represents the gray scale range: black at the left end and white at the right end, with levels of gray in between. The number of pixels registering a particular gray level determines the height of each bar along the axis.

A Coomassie blue-stained protein gel visualized with a white light box has a histogram reflecting mostly bright pixels:



Histogram of a typical Coomassie gel

Most of the pixels are found in the light portion of this histogram. The dark bands represent a small number of pixels and include a variety of gray values, and therefore do not show up as a single peak.

The histogram function is particularly useful to verify that an image spans the maximum range of gray levels. When an image is to be used for analysis, it is especially important that the gray level range be as large as possible. If an image does not include most of the gray levels, we recommend repeating the image capturing process.

The Rotate / Flip Tool

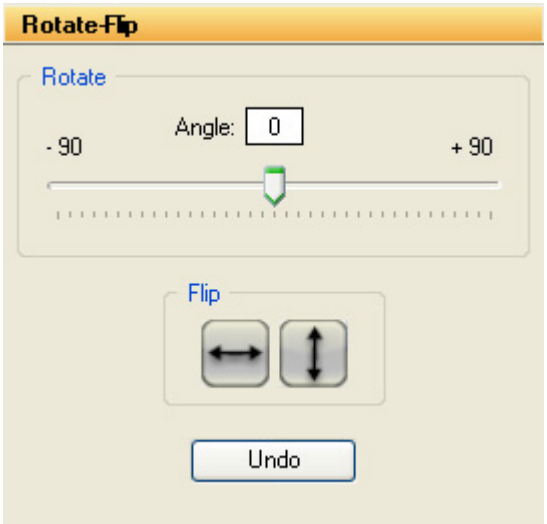
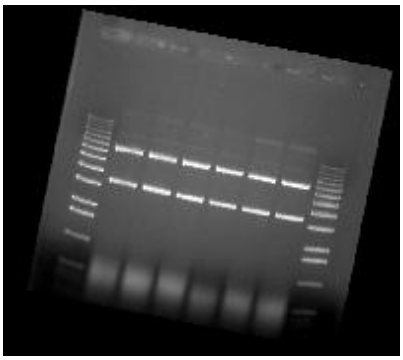


Figure 4.3 The Rotate / Flip Tool

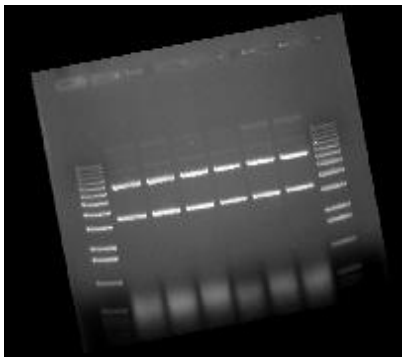
The Rotate / Flip tool is found in Tool Box, Enhancement Tools. This function rotates the image in a clockwise or counterclockwise direction by 1 degree increments up to a maximum of 90 degrees in either direction. This is a useful tool if the image is not aligned properly during the capturing process.

To rotate an image, click and hold down on the center sliding bar with the left mouse button and move it left or right until the desired angle of rotation appears in the rotate box. Release the left mouse button and image will rotate to the desired angle. To undo a rotation, just click on the Undo button. Also, a Flip option allows for the image to be rotated 180 degrees in a vertical or horizontal fashion.

The Reset button on the main software interface will also remove any rotations or image flips and return to the display to the original image



Rotated 11 degrees clockwise



Rotated -11 degrees

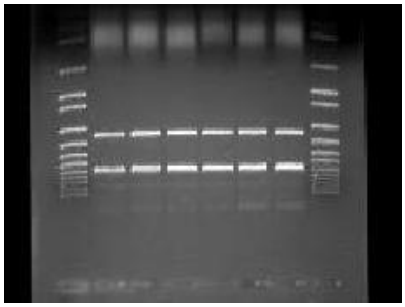
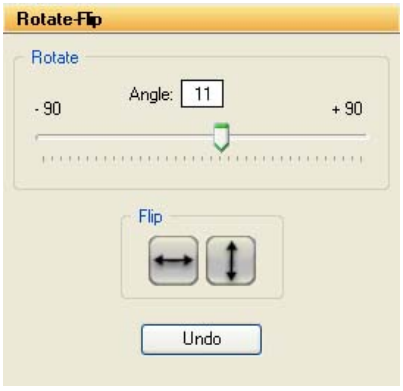
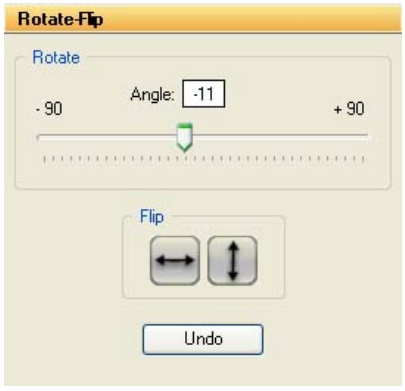


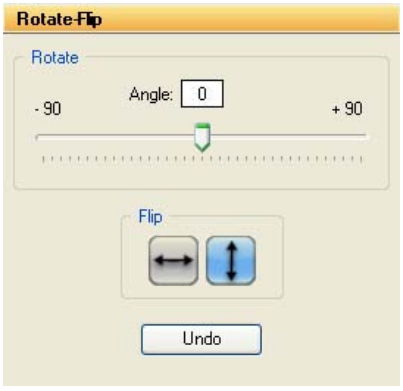
Image flipped vertically (Counterclockwise)



Rotate / Flip box at 11 degrees



Rotate / Flip box at –11 degrees



Rotate / Flip box with vertical Flip button pressed

Annotations

The annotation tools, found in ToolBox, Enhancement Tools, include a number of different options for adding text (including Greek symbols), drawing arrows and otherwise marking an image. Note that these tools are for annotation only. For information on drawing objects for quantitation purposes, see Chapter 5.

Figure 4.4 Annotations Toolbox

Object Attributes

Use the COLOR, PEN WIDTH, PEN STYLE, LINE ENDS, TEXT STYLE and/or TEXT ORIENT menus to specify object attributes. Attributes can be assigned to the cursor before drawing or typing. Alternatively, they can be assigned to an object while it is in "edit" mode (see the following pages for more details).

Annotation Colors

Annotations can be displayed in a variety of colors. The color options are displayed by clicking the COLOR radio button. To select a color, simply click the cursor on the button labeled with the desired color. The color button appears depressed, indicating that it is selected. Any annotations subsequently entered will appear in that color. It should be noted, however, that annotations are printed in gray scale on the video printer. Further, when an image is saved as a modified image, the annotations are saved in gray-scale, not color.

Line Thickness

The PEN WIDTH menu specifies the thickness of lines when using the freehand, lines, box and circle drawing tools. Click on the appropriate checkbox for the desired width. All annotations subsequently entered will appear at that width.

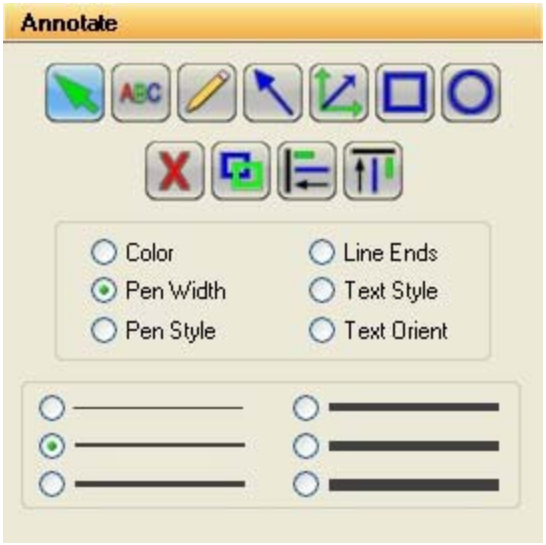


Figure 4.5 Pen Width Selection Tools

Line Types

The PEN STYLE menu specifies the style of lines when using the freehand, lines, box and circle drawing tools. Click on the appropriate checkbox for the desired style. All annotations subsequently entered will appear in that style. Note: these pen styles only work with a thin line (see ***Line Thickness*** above).

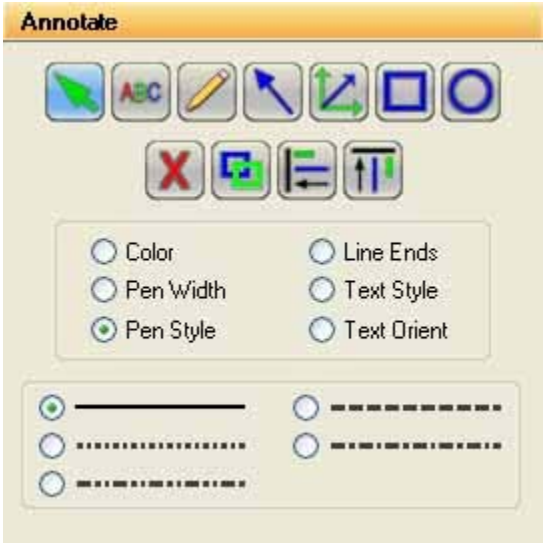


Figure 4.6 Pen Style Selection Tools

Arrows and Straight Lines

The LINE ENDS menu specifies the style of the ends of straight lines (no arrow, single arrow or double arrow). Click on the appropriate checkbox for the desired style. Note: these line ends work with any line thickness.

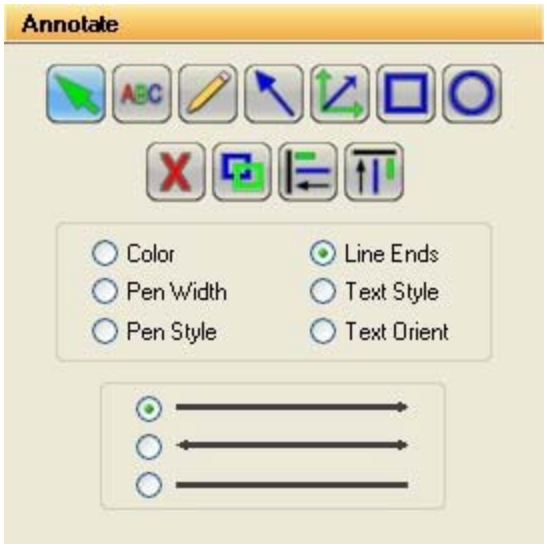


Figure 4.7 Line Ends Selection Tools

Text Background and Font

The TEXT STYLE menu specifies the style of text. Click on the appropriate checkbox to show text with or without a background. An opaque background is useful if annotations will be made on an image that has wide variations in gray scale. By using an opaque background, text will not be "lost" in the background of the image.

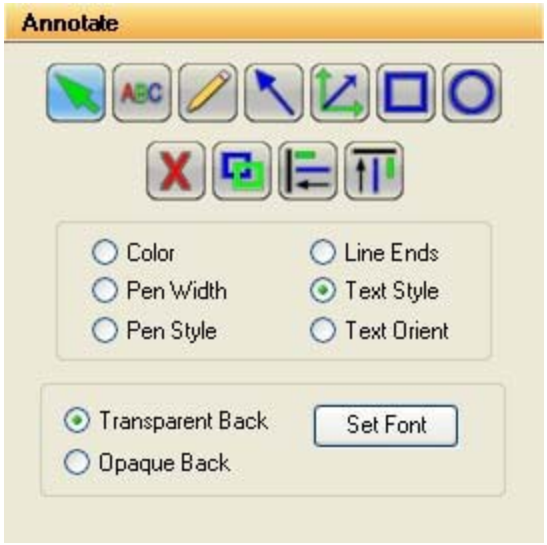


Figure 4.8 Text Style Selection Tools

This is also the window in which specific font is chosen. When the Set Font button is depressed, a selection box appears, from which text style can be chosen.

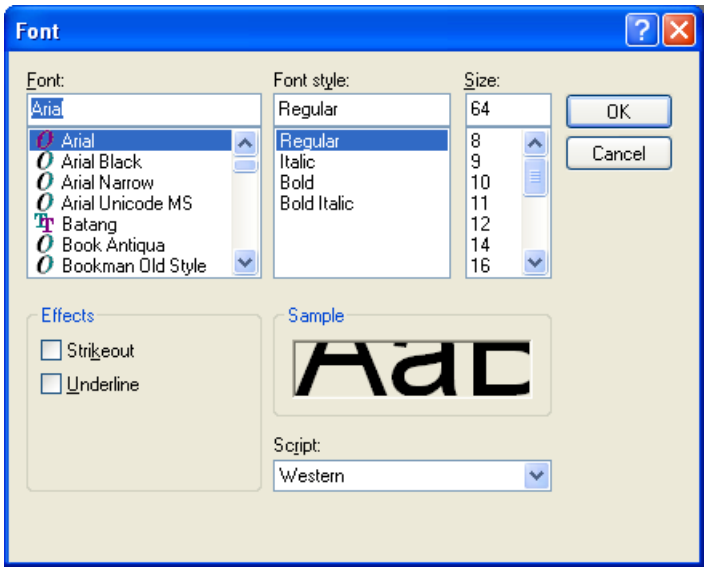


Figure 4.9 Font Selection Window

Note:
To choose Greek symbols (such as α, β, λ, π, and θ) choose the Symbol font:

a b c d e f g h i j k l m n o p q r s t u v w x y z

α β χ δ ε φ γ η ι φ κ λ μ ν ο π θ ρ σ τ υ ϖ ω ξ ψ ζ

Text Angles

In the TEXT ORIENT window, select whether text should be oriented vertically, horizontally, or at an angle (in 15° increments).

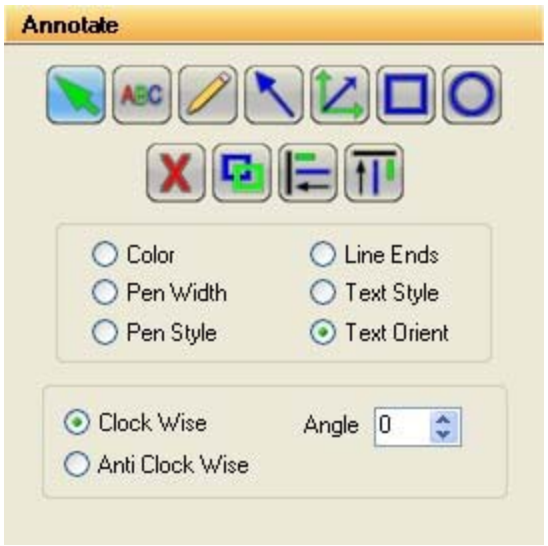


Figure 4.10 Text Orient Selection Tools

Note: only rotate fonts that are True Type (indicated by TT in front of the name); other fonts (such as Courier and Fixedsys) do not re-scale properly, giving unpredictable results.

The Drawing Tools

Once the object attributes have been defined, click the cursor on any of the drawing tool buttons to assign the function associated with that button to the mouse. The cursor will change from an arrow to a cross, indicating that AlphaView is in “drawing” mode.

After selecting a drawing tool, move the cursor to the correct position on the image to begin drawing. Press and hold the left mouse button and move the mouse to the end point of the object to be drawn. Release the left button, and the object should now appear.

Boxes will appear at the corners of the new object and the cursor will revert to an arrow, indicating that AlphaView is now in “edit” mode. At this point, the object can be resized or repositioned. The color, pen thickness, line type, etc. can also be changed, simply by clicking on the desired choice (as described in ***Object Attributes*** above).

To draw another object, click the right mouse button to return to “draw” mode, or click on one of the drawing tool buttons.

 The selection tool, allows the user to select all drawing for further operations, such as vertical alignment.

The button labeled with an "ABC" adds text to the image. Place the cursor at the location on the image where the left edge of the text should appear. Click the left mouse button and begin typing. To place another piece of text, click where it should be placed. Once all text is entered, click on the right mouse button. To edit text, double-click on it. An edit window will appear, in which changes can be made. To change fonts, see ***Text Background and Font*** above.

 The button labeled with a pencil icon allows the user to draw lines freehand. After clicking on the pencil, move the cursor to the correct position on the image to begin drawing. Press and hold the left mouse button. Using the mouse, move the cursor as if it were a pencil. When finished drawing, release the mouse button.

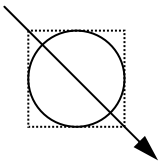
 The button labeled with a diagonal line and arrow, draws arrows and straight lines. After clicking on the button, move the cursor to the position on the image where the line should begin. Press and hold the left mouse button. Using the mouse, move the cursor to the other end point of the line then release the mouse button. The arrow can be adjusted by clicking on one of the boxes at the end (the other will serve as an anchor point) or by clicking in the middle to drag the entire arrow.

 The button labeled with an angles arrows on its side is a line drawing tool, very similar to the one described above. The significant difference is that this tool limits the angle that the line can be drawn to increments of 45°.

 The button labeled with a square draws a rectangle or square of any size on the image. After clicking on the button, move the cursor to the position that should correspond to one of the corners of the rectangle. Press and hold the left mouse button. Using the mouse, move the cursor to enlarge the rectangle. When it reaches the desired size, release the left mouse button.

The button labeled with a circle draws a circle of any size. After clicking on the button, move the cursor to the position on the image where the circle should be started. Press and hold the left mouse button. Using the mouse, move the cursor to enlarge the circle. When the circle reaches the desired size, release the left mouse button.

Hint: to draw a perfect circle around a portion of an image, first visualize a square surrounding the area of interest. Position the mouse in the upper left hand corner of the square. Click and drag the mouse down across the area of interest at a 45° angle until the circle encloses the area of interest.



Sample Annotations

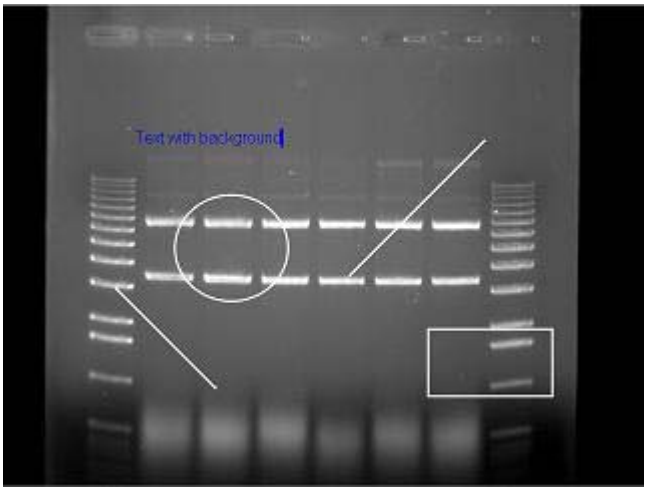


Figure 4.11 Sample Annotations

Annotated image showing: freehand drawings, lines with various characteristics, circles, squares, text with various characteristics

The Editing Tools

When the cursor is in “edit” mode, it can be clicked on an object to select it. (Note: the cursor can be toggled between “edit” and “drawing” modes by clicking the right mouse button.)

When an object is selected, small square boxes appear at the corners. Selected objects can be resized, copied, deleted or moved:

- To resize an object, click on one of the gray boxes at the corners of its perimeter and drag the box until the object reaches the desired size.
- To copy an object, use the Copy tool.
- To delete an object, use the Cut tool or the Eraser.
- To move an object, click within its boundary and drag it into the desired location.

To select more than one object, outline them with the mouse; any objects that fall completely within the outline drawn will be selected. (Note: The entire object must be enclosed by the cursor’s movement in order to be selected.)

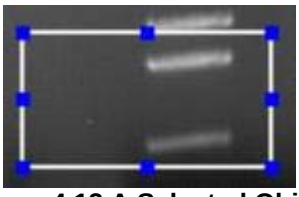
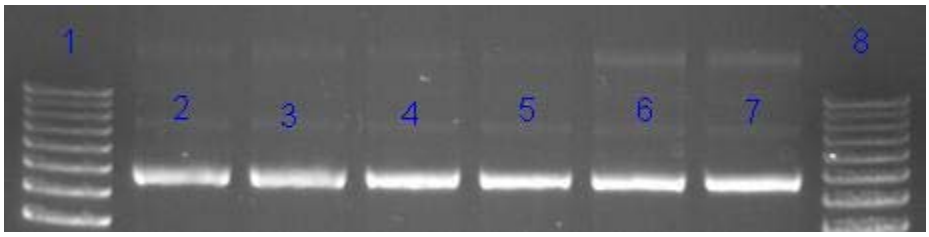


Figure 4.12 A Selected Object

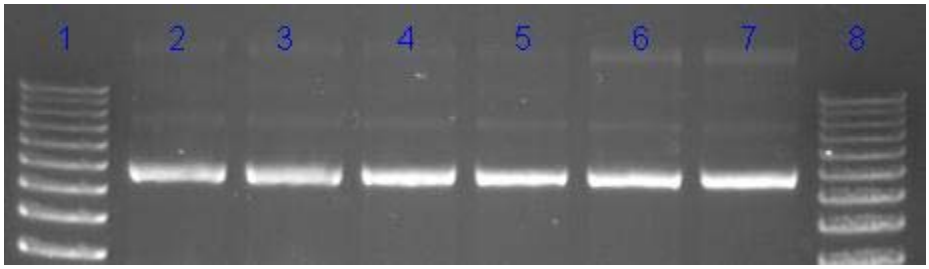
Once an object or group of objects has been selected, clicking on the Cut tool deletes it from the image.

The Copy tool makes an exact copy of the selected object. The new object becomes the selected object, and can be repositioned by placing the cursor within the object's boundary and moving it to the desired location.

The Horizontal Alignment tool aligns annotations in a straight horizontal line. This is especially useful for labeling lanes, etc. To use this tool, draw text on the image, select it, click the Horizontal Alignment tool, then deselect the text. The text will now be aligned in a straight line across the image.



Text Prior to Horizontal Alignment



Text After Horizontal Alignment

The Vertical Alignment tool aligns annotations in a straight vertical line. This is especially useful for labeling markers, etc. To use this tool, draw text on the image, select it, click the Vertical Alignment tool, then deselect the text. The text will now be aligned in a straight line down the image.

False Color

These tools consist of eleven pre-defined color palettes that can be applied to an image. To select a palette, simply click on one of the four buttons labeled GRAY PALETTE, HIGH-LOW, NEXT or PREVIOUS.

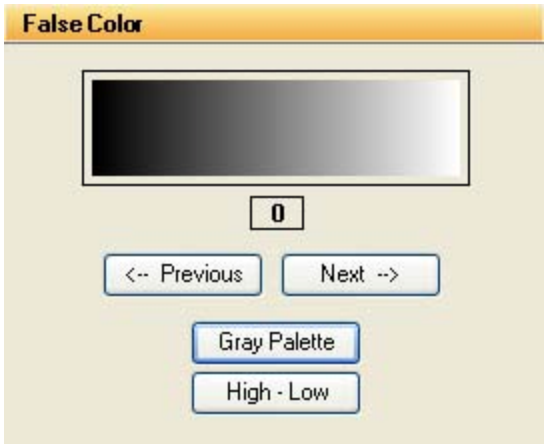


Figure 4.13 False Color Selection Box

When a palette is selected, its range of colors is displayed to the left of the palette buttons and automatically applied to the image. To apply a different palette to the image, click the NEXT or PREVIOUS buttons.

Note: changes in Black level, White level and Gamma setting can alter the effect of each of the palettes, and can enhance the results produced.

Gray Scale (Palette 0)

This is the default or standard gray scale, consisting of different gray levels, ranging from black to white.

High - Low (Palette 1)

This is a modified gray scale palette in which black is replaced with green, and white is replaced with red. Over- and under-exposed areas of the image are thus shown as green or red, while areas within the linear range of the CCD chip are shown in gray scale. The Saturation Palette is especially useful during quantitation, as areas outside the linear range of the instrument do not give accurate quantitative information. This palette allows the user to avoid those areas during quantitative analysis.

This palette can also be accessed by clicking the Show Saturation checkbox in the Camera Setup and Preview function (accessible by clicking on the Camera icon in the ToolBar window)

Other Palettes (Palettes 2 through 11)

These are color substitution palettes in which the gray levels are translated into different color ranges. These palettes can be useful to help distinguish features and highlight details on an image.

Palette 2 maps the gray scale levels to a red/green/blue palette. Values of 0 are mapped to red; saturated to blue, and values in between to green.

Palette 3 maps the gray scale levels to a red/green/blue palette. Values of 0 are mapped to blue; saturated to green, and values in between to red.

Palette 4 maps the gray scale levels to a red/green/blue palette. Values of 0 are mapped to green; saturated to red, and values in between to blue.

Palette 5 maps the gray scale levels to a cyan/magenta/yellow palette. Values of 0 are mapped to cyan; saturated to yellow, and values in between to magenta.

Palette 6 maps the gray scale levels to a cyan/magenta/yellow palette. Values of 0 are mapped to yellow; saturated to magenta, and values in between to cyan.

Palette 7 maps the gray scale levels to a cyan/magenta/yellow palette. Values of 0 are mapped to magenta; saturated to cyan, and values in between to yellow.

Palette 8 maps the gray scale levels to shades of red palette. This palette may be useful when viewing red color images.

Palette 9 maps the gray scale levels to a red palette. Values of 0 are mapped to dark red; saturated to white, and values in between to shades of red.

Palette 10 maps the gray scale levels to shades of blue palette. This palette may be useful when viewing blue color images.

Palette 11 maps the gray scale levels to a blue palette. Values of 0 are mapped to dark blue; saturated to white, and values in between to shades of blue. This palette may be useful when printing an image of a Coomassie-stained protein gel onto a color printer.

Palette 12 maps the gray scale levels to shades of green palette. This palette may be useful when viewing green color images.

Palette 13 maps the gray scale levels to a green palette. Values of 0 are mapped to dark green; saturated to white, and values in between to shades of green. This palette may be useful when printing an image of a SYBR® Green I-stained protein gel onto a color printer

Palette 14 maps the gray scale levels to an orange palette. Values of 0 are mapped to dark orange; saturated to white, and values in between to shades of orange. This palette may be useful when printing an image of an EtBr-stained gel onto a color printer.

Image Filters

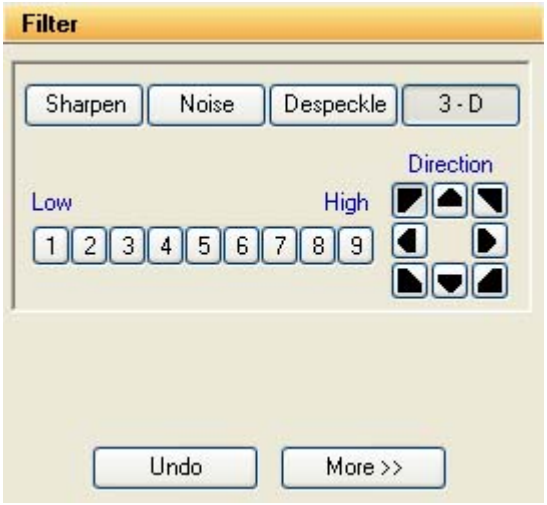


Figure 4.14 Filters Toolbox with 3-D (contour) selected

AlphaView includes a variety of enhancement filters that can improve the appearance of an image. Some filters sharpen detail, others smooth and reduce random noise. Still others help visualize edges and separate closely spaced bands or objects. Depending upon the unique characteristics of an image, the results of each filtering operation vary. Assess the characteristics of the image and then select the filter designed to minimize its imperfections.

When an image is filtered, the original image information is replaced with the results of the filtering operation. As a result, the original image information is altered. To avoid losing the original image, save it as an original TIFF file *before* applying a filter.

When the FILTERS button in ToolBox, Enhancement Tools is selected, a pop-up box appears. If the desired filter is not shown, choose MORE to display more filters. One or many filters can be applied to a single image.

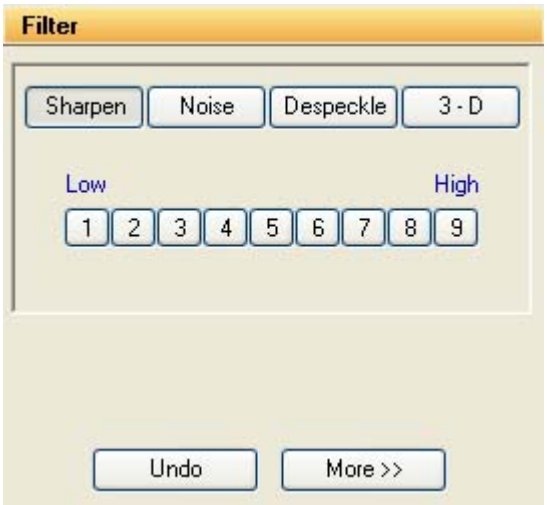


Figure 4.15 The Filters Toolbox with Sharpen highlighted

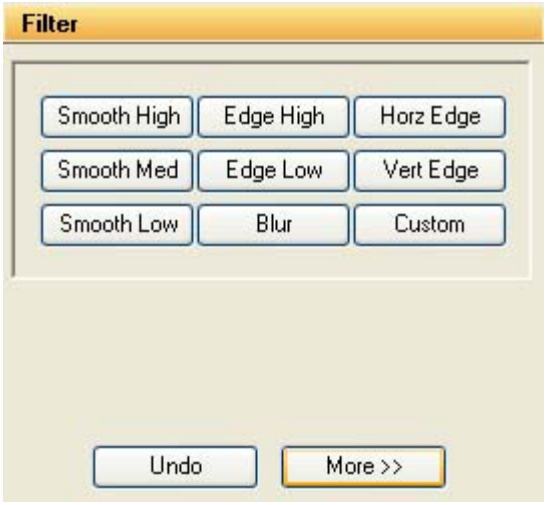


Figure 4.16 The Filters Toolbox with More selected

General Information

To enhance an image, filters change the value assigned to each pixel. The new value assigned to a pixel is determined based on the values of the other pixels in its local vicinity (or "neighborhood"). The neighborhood is a two-dimensional matrix of pixel values, where each dimension has an odd number of elements. The "pixel of interest" is the one at the center of the neighborhood. This is the pixel whose old value is being replaced with a new one as the result of the filtering algorithm.

The pixels in a neighborhood provide information about the brightness trend. This information is important to the filtering process. The brightness trend is also referred to as the "spatial frequency." Images with high spatial frequency content contain large, closely spaced changes in pixel values. For example, on a black and white checkerboard, the smaller the squares, the higher the frequency content.

Images with low spatial frequency content (for example, images of clouds) contain large areas of slowly changing pixel values.

Most of the filter options available (with the exception of the Noise filters) use a weighted summation process to determine the value assigned to the pixel of interest. Each pixel in a 3x3 neighborhood is multiplied by a "convolution kernel" having the same dimensions. The resulting sum is assigned to the pixel of interest.

			(K1xP1)+ (K2xP2)+ (K3xP3)+ (K4xP4)+
P1 P2 P3		K1 K2 K3	(K5xP5)+
P4 P5 P6	X	K4 K5 K6	(K6xP6)+
P7 P8 P9		K7 K8 K9	(K7xP7)+ (K8xP8)+
3x3 pixel neighborhood (P5 is being calculated)		convolution kernel	(K9xP9) **New Value for P5**

Each element of the convolution kernel is a weighting factor, also called a "convolution coefficient." The size and arrangement of these weighting factors determine the type of transformation the image will undergo. Changing a weighting factor influences the overall sum and, therefore, affects the value given to the pixel of interest.

Sharpening Filters

These filters can increase image sharpness and provide edge enhancement. However image noise may be enhanced as well. These filters accentuate the high-frequency details of an image while leaving the low-frequency content intact. High frequency portions of the image get brighter while low frequency portions become black.

Sharpen level 9 (high) has the largest effect on the image. Sharpen level 5 has an intermediate effect. Sharpen 1 (low) has the most subtle effect on the image. 9 different sharpening levels are available for optimization of the image.

Noise Filters

This filtering process uses the values of the pixels contained in the area surrounding a pixel to determine the new value given to the pixel of interest. The noise filter sorts the pixels in the neighborhood into ascending order and picks the middle or median pixel value as the new value for the pixel of interest. 3 levels of noise reduction are available.

Despeckle Filters

The despeckle filter is a type of smoothing filter based on data rejection. Pixels in the neighborhood (usually the adjacent pixels) are used as a data set upon which the average and standard deviation of the set are calculated. If the pixel of interest (the center of the neighborhood) is different from the neighborhood average (either greater or lesser) by a threshold (a multiple of the standard deviation) it is replaced by the average value.

The effect of the filter is to "smooth" pixels that are much different from their neighbors. Artifacts such as hot pixels, cosmic rays etc. are commonly rejected by this type of filter. The filter strength is controlled by the threshold factor. For large factors very little data is rejected as only very large deviations are required for rejection, where as, low thresholds result in more smoothing. (For example, setting #1 results in outliers of 1 standard deviation greater or lesser than the neighborhood average to be corrected for, setting #2 results in outliers of 2.5 standard deviations to be corrected for and setting #3 results in outliers of 5 standard deviations to be corrected for.)

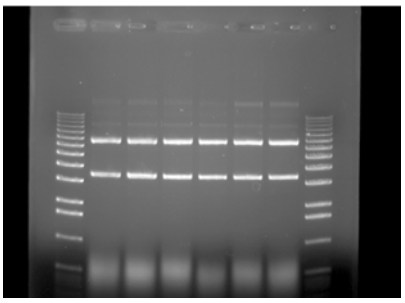
These filters are particularly effective in eliminating random noise contained in an image and produce less blurring than the Noise filter described above.

3-D (Contour) Filters

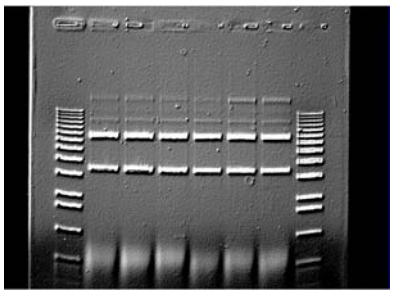
These filters are particularly useful for visualizing faint bands. They produce a "3-D" effect, defining edges and making details easier to see.

3-D level 9 (high) has the largest effect on the image. 3-D level 5 has an intermediate effect 3-D level 1 (low) has the least effect on the image. 9 different 3-D levels are available for optimization of the image.

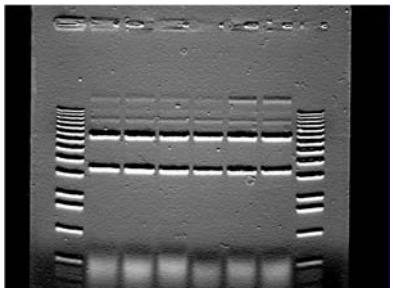
In addition, 3-D allows the user to control the 'direction' of the 3-D shadowing effect. Once the level is refined, just click on a direction arrow to visualize the shadowing adjustment. The default shadow direction is in the lower right hand corner direction and 9 different directions are available.



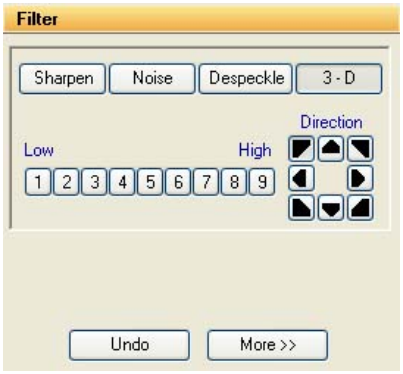
Original Image



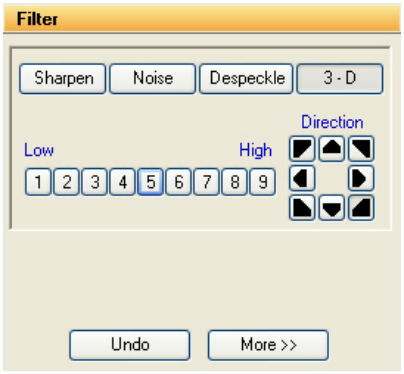
3-D Image Level 5 & lower right shadow



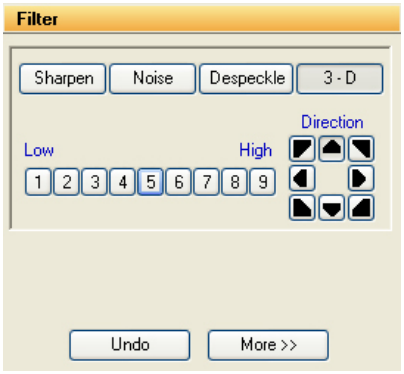
3-D Image Level 5 & upper middle shadow



Original Filter Toolbox



Lower right shadow



Upper middle shadow

Smoothing Filters

These filters are very useful for reducing the visual noise present in an image. When a smoothing filter is applied to an image, rapid changes in intensity are averaged out with the remaining pixels in the neighborhood, thereby decreasing the high frequency content. The visual result is a slight smoothing of the image because sharp pixel transitions are averaged with their surroundings.

Smooth High has the largest effect on the image. Smooth Med has an intermediate effect. Smooth Low has the most subtle effect on the image.

Edge Filters

These filters use Laplacian enhancement to highlight edges, regardless of direction. All edge-enhancement operations attenuate the low frequencies of the image. Regions of constant intensity or linearly increasing intensity become black as a result of these transformations, and regions of rapidly changing intensity values are highlighted.

Note: The White level may need to be adjusted after using the Edge filters in order to see the result of this filtering process.

Horizontal Edge Filter

This filter brightens horizontal edges. This can be useful in pinpointing bands on a gel. The horizontal edge filter (Horz. Edge) enhances image edges by shifting an image vertically by one pixel and then subtracting the shifted image from the original. In an area of constant pixel intensity, the subtraction yields black pixel values. At an edge, which is an area with large changes in intensity, the subtraction yields light-colored pixel values. The larger the difference in intensities, the lighter the resultant pixels.

Note: After applying the horizontal edge filter the entire image may appear black, and might require reducing the White level in order to better visualize the results.

Vertical Edge Filter

This filter (Vert. Edge) brightens vertical edges using the approach described for the horizontal edge filter (see above), except that the image is shifted horizontally before the shifted image is subtracted from the original. In this case, the vertical edges produce light-colored pixel values. As in the horizontal edge filter, it may be necessary to adjust the White level in order to better visualize the results of this filtering process.

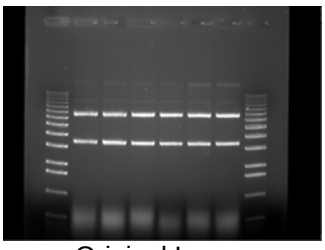
Custom Filter

This function also allows the user to customize filters to his/her own specifications. Using the weighting factors of the other filters as a frame of reference, experiment with new weighting factor values.

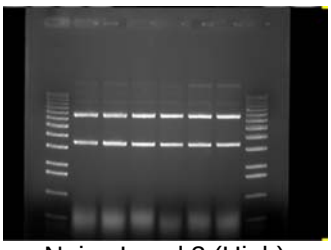
The UNDO Button

The upper right button is labeled UNDO. This reverses the last filtering process applied to an image. To revert the image to its original state after multiple filters have been applied, press the RESET button on the main interface.

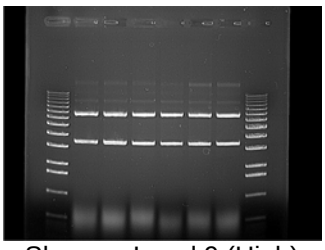
Examples of Filter Results



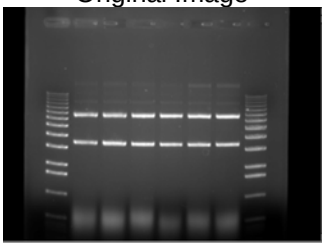
Original Image



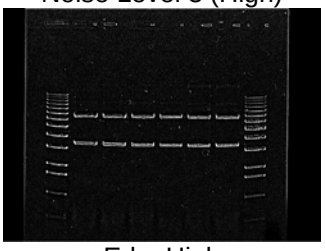
Noise Level 3 (High)



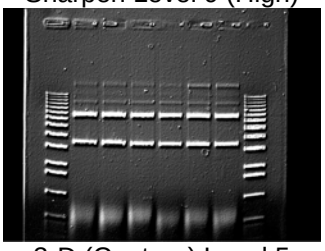
Sharpen Level 9 (High)



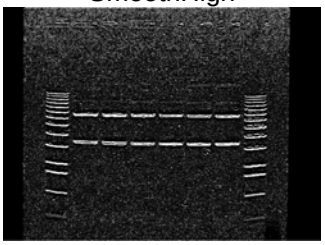
SmoothHigh



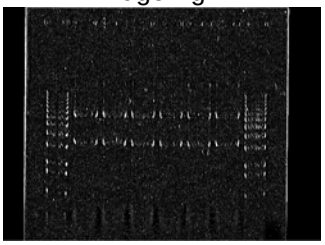
EdgeHigh



3-D (Contour) Level 5



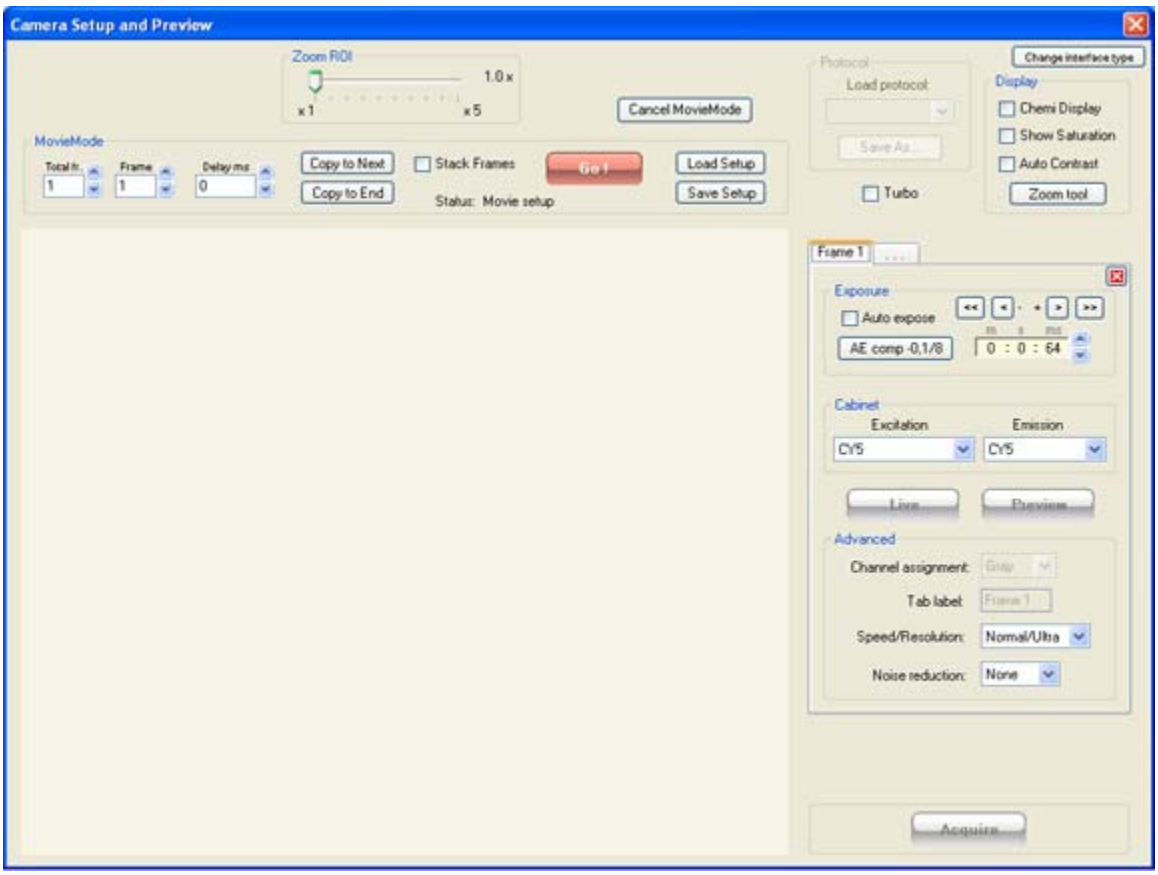
Horizontal Edge



Vertical Edge

Movie Mode

If kinetic, multiplex, color, or chemiluminescence experiments are desired where you wish to have the system automatically capture several images at preset exposure times, preset time delay between images, preset lighting sources, and preset filter choices, the MOVIE box can be found in camera setup acquisition screen. To access this screen select the acquire button on the tool bar and then select 'Movie Mode' on the acquisition screen. AlphaView stand alone software does not include Movie acquisition tools)



Once **MOVIE MODE** is selected, a display box will appear for independent control of all lighting, filters, and exposure delay for each image frame.

The **TOTAL FRAMES** setup provides you with the ability to determine how many individual frames (images) you want for the movie. There is a maximum of 50 frames (images) and a minimum of 1 frame that can be captured with each movie.

The **FRAME** selection is used for setting up the conditions for each frame (image). For example, if three (3) images are to be captured, you would choose FRAME 1 and setup all of the desired lighting and filter requirements. You can then click on FRAME 2 and repeat the above. Or, you can click on **COPY TO NEXT** to help speed up the setup process. **COPY TO NEXT** copies all settings from the previous frame to the current frame. Usually, for chemiluminescence imaging, all lighting is off and the filter wheel is positioned for the chemiluminescence position for all frames. Thus, the only variable that is changing from one frame to the next is the exposure time. In this situation, **COPY TO NEXT** is a useful tool to save time in the setup process.

If you are performing kinetic experiments where you want to have a predetermined delay between captured images, then you can use **EXP DELAY** to configure this function. The default **EXP DELAY** is set for the shortest possible delay (19 milliseconds), but can be configured up to 50 minutes between each image. Also, if your exposure delay and/or exposure time and/or lighting options/filter position is consistent for the entire movie, then once you setup the first frame, you can select the **COPY TO END** selection to automatically choose the first frame settings for the entire movie of frames (images).

Once the movie is setup to the desired configuration, click on the **GO** button. The movie will then begin the image acquisition for each frame of the image. When it is complete, the movie setup box will disappear and the TOOLBOX window will automatically configure to the MOVIE tools. This will allow you to playback the movie, save or load the movie, or record a new movie.

Once all images have been captured, the above Movie display box will become displayed. The remaining buttons will perform the following tasks:

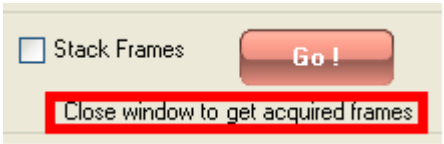
REC	Move to Camera Setup and Preview, Movie Record setup functions to record a movie.
PLAY	Display a continuous loop 'movie' of all of the captured images.
STOP	Stop the movie at the current frame display
PAUSE	Pause the playback of the movie at a user defined image
REW (rewind)	Rewind the movie to the first image
REV (reverse)	Play the movie in a continuous loop in reverse
FWD (forward)	Forward the movie to the last image
DEL (delete)	Delete the movie on the display. THIS FUNCTION WILL NOT DELETE A MOVIE SAVED TO THE HARD DRIVE.
LOAD	Load a previously saved movie. THIS FUNCTION WILL NOT LOAD INDIVIDUAL IMAGES PREVIOUSLY CAPTURED IN NORMAL CAPTURE MODE.
SAVE	Save a movie of images.

Saving An Individual Image From a Movie

After you load, play, and stop a movie at the desired image, it is possible to save the individual image seen on the screen. Use the SAVE AS button located on the tool bar to save the image in the desired location and file format on the local or network drives.

Saving Partially acquired movie

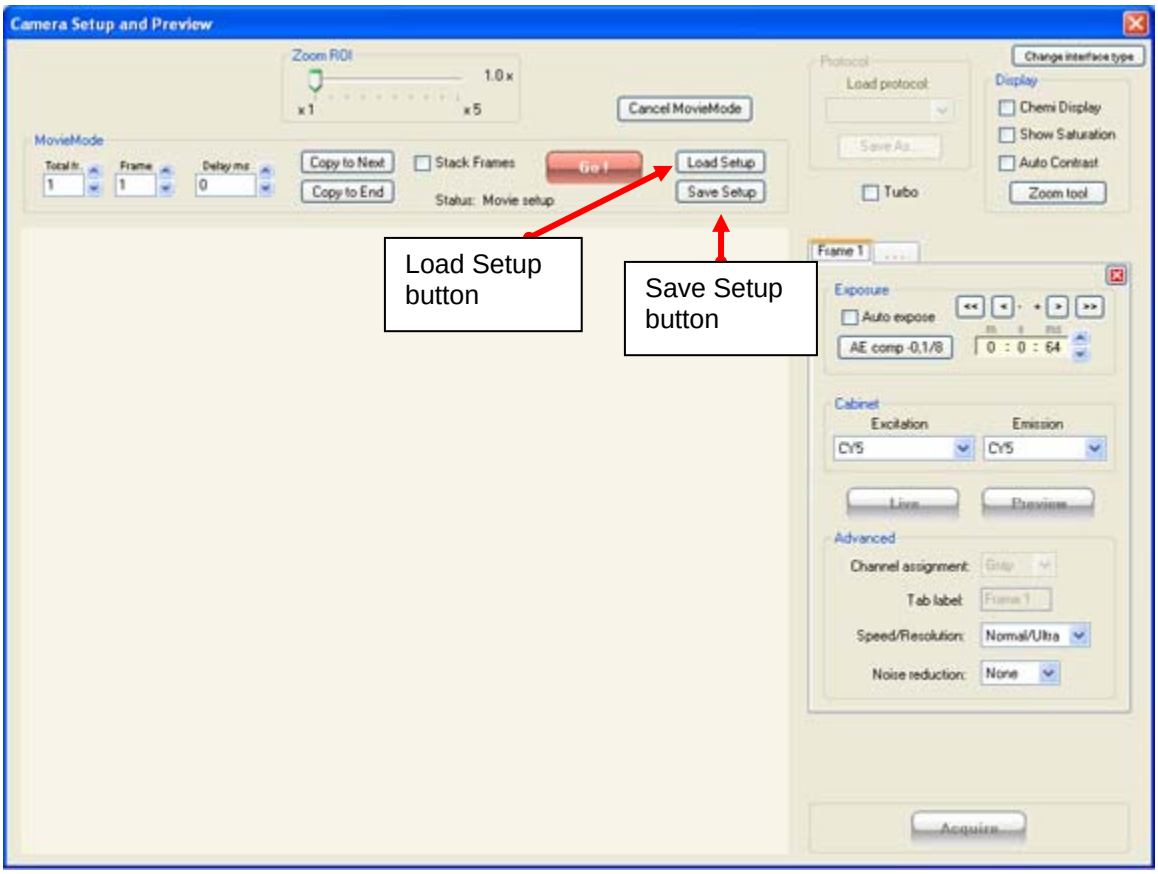
It is possible to stop the movie in the middle of acquiring frames, the user can exit the movie mode to save the acquired frames only.



Option 1: Go to continue movie.
Option 2: Close Window to view and/or save acquired frames.

Movie Mode: Save/Load Movie Mode setup routines

Two buttons “Save Setup” and “Load Setup” allow you to save and load all Movie Mode setup parameters. Files are saved as *.mvf files.



Frame Stacking

At the top of the Movie Set-up window is an option for stacking frames. If this selection was chosen during acquisition of the image Stack Frames will use all previous exposure information to sequentially add images to one another. Normal Sequence will not perform this addition. Please note that stacking frames will increase the noise level in acquired images.

Sample Case

Sample case:

Capture 5 frames at 1-5 sec exposure for total time exposure time of 15 sec

Display after summation of following frames:

Frame 1 = Image (1 sec exp time)

Frame 2 = Frame1 + Image (2 sec exp time)

Frame 3 = Frame2 + Image (3 sec exp time)

Frame 4 = Frame3 + Image (4 sec exp time)

Frame 5 = Frame4 + Image (5 sec exp time)

Chapter 5. THE IMAGE ANALYSIS TOOLS

Default Analysis Tools

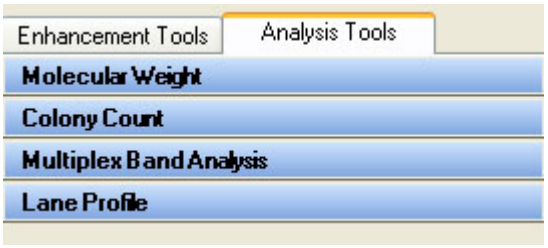


Figure 5.1 Default Analysis Tools in the ToolBox

Default image analysis tools are contained within the Tool Box as indicated. This tool set allows the user to perform molecular weight determinations, automatic counting of colonies and cells, multiplex band analysis and lane profile densitometry.

Molecular Weight Determination

Introduction

The button in ToolBox, Analysis Tools, labeled MOL. WEIGHT, opens a set of tools for entering the values of known molecular weight markers and determining the molecular weights of unknown bands on the image.

When the MOL. WEIGHT button is selected, a function box appears in the area to the lower left of the screen, and a data box appears on the image. (If the data window obscures part of the image containing bands of interest, it can be moved.)

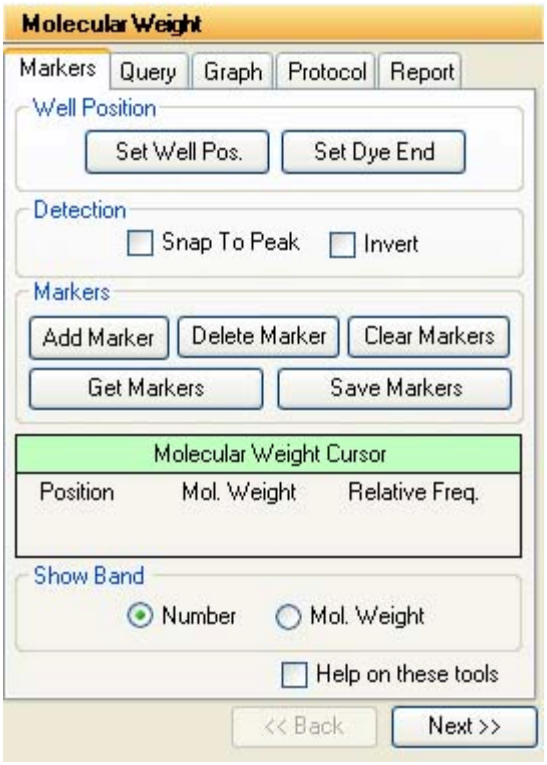


Figure 5.2 Molecular Weight Tools

Molecular Weight Results

Export

MARKERS

Band	Position	Mol. Weight	Rf
1	238	14.00	0.496
2	256	52.00	0.533
3	280	43.00	0.583
4	314	24.00	0.654

QUERIES

Band	Position	Mol. Weight	Rf
1	240	18.22	0.500
2	260	50.50	0.542
3	283	41.32	0.590
4	317	N/A	0.660

Figure 5.3 Molecular Weight Data Box

At the top of the data box is a bar with two menus: Marker and Query. The area below the menu bar is divided into two sections. Molecular weight marker data is displayed in the upper section and the calculated or query data is displayed in the lower section.

Entering Known Molecular Weights for Markers

An unlimited number of molecular weight standards can be defined, either all in one lane, or in multiple lanes. To input markers, open the Marker menu and select Add Marker. **Note: to enter standards from previously-saved files, see *Applying a Set of Saved Markers* below.**

Notice that the cursor flashes and has a short, horizontal line associated with it. Move the cursor to the lane containing the molecular weight standards. Beginning at the top of the lane, position the cursor so the horizontal line is aligned with the band.

If the SNAP TO PEAK function has been activated, the cursor jumps to the highest pixel value in the band as the cursor approaches it. If bands are so tightly spaced that the line is not coinciding with the band, turn off SNAP TO PEAK and place the marker on the band manually.

When the horizontal line is positioned correctly, click the left mouse button. A dialog box appears requesting the band's molecular weight. Using the numeric keypad in the dialog box (or the numbers on the keyboard), enter the known molecular weight. Alternatively, enter the value using the keyboard.

After entering the molecular weight, press either the OK button in the dialog box (or the <**Enter**> key on the keyboard). The dialog box disappears, the number of the band appears on the image, and the band's data is added to the Markers section of the data box. The horizontal red line remains, indicating that the cursor is ready to select the next band as described above.

After entering the value for the last marker, click the Add Markers Complete button instead of OK. This will deactivate the value-entering function and will return the cursor to its normal mode. (To reactivate the value-entering function, select Add Marker from the menu again, or click the right mouse button.)

The Molecular Weight Data Box

The data box is located near the top of the image. Initially, no values are displayed in the data box. When a molecular weight value is assigned to a band, the following information is displayed in the data box:

- Band numbers are assigned beginning with the first band, continuing in the order selected.
- The position corresponds to the band's location along the y-axis, ranging from 0 to 1030.
- The value displayed under the Mol. Wt. heading is the molecular weight value entered for a known band or the value calculated for an unknown band.
- The R_f value for each band is also displayed. Unless otherwise specified, AlphaView assumes the origin (R_f value = 0.00) is located at the top of the screen, and dye front (R_f value = 1.00) is at the bottom of the screen. Using these points as a frame of reference, AlphaView calculates R_f values for the intermediate bands.

Molecular Weight Marker and Query Data

If the data box obscures part of the image, it can be resized or moved using Windows® functions, or it can be hidden using the Hide Data checkbox.

Repositioning and Deleting Markers

Marker band indicators can be repositioned simply by positioning the cursor, clicking, and dragging the line to the desired location.

To delete a molecular weight marker, point the cursor at the appropriate marker band and click the left mouse button. This highlights the band in question by putting a red box around it. (Alternatively, clicking in the data table highlights the marker's information and selects the band on the image.)

Click on the Delete Marker function in the Marker menu. The marker is deleted, as is the band's data in the marker data table. Markers entered after the deleted bands are renumbered on the image and the data table.

To delete all the markers and start over, select the Clear Markers function in the Marker menu.

Determining Molecular Weights of Unknown Bands

After marker values are entered, the molecular weight of any unknown band can be determined.

Manually Selecting Bands

To indicate unknown bands manually, select the Add Band function from the Query menu. Just as in Add Marker above, a line will appear attached to the cursor. Point the cursor at the band of interest and click the mouse button. The molecular weight of the band is automatically calculated and displayed in the Queries section of the data box.

To select a second band, click the right mouse button and the cursor line will reappear. Repeat this procedure for all bands for which molecular weights is to be determined. Note that the molecular weight markers appear in blue while the queries appear in green.

The molecular weight of a band is calculated based on the graph of the known marker bands. (Note: If a query band lies outside of the markers, it will be extrapolated in “Least Squares Fit” mode or given a value of “N/A” in “Point-to-Point” mode.) See ***The Graph Tool*** below for more information.

Automatic Band Finding
AlphaView includes an algorithm for automatically finding bands in query lanes. To use this function, select Auto Query from the Query menu. A yellow vertical line will appear attached to the cursor. Position the line over the lane of interest and click the left mouse button. The bands in the lanes will be selected automatically, and their data will appear in the Data box.

Repositioning and Deleting Bands
Bands can be repositioned simply by positioning the cursor, clicking, and dragging the band to the desired location.

To delete an unknown band indicator, point the cursor at the band and click the left mouse button. The band in question is highlighted. (Alternatively, clicking in the data table highlights the band’s information and selects it on the image.)

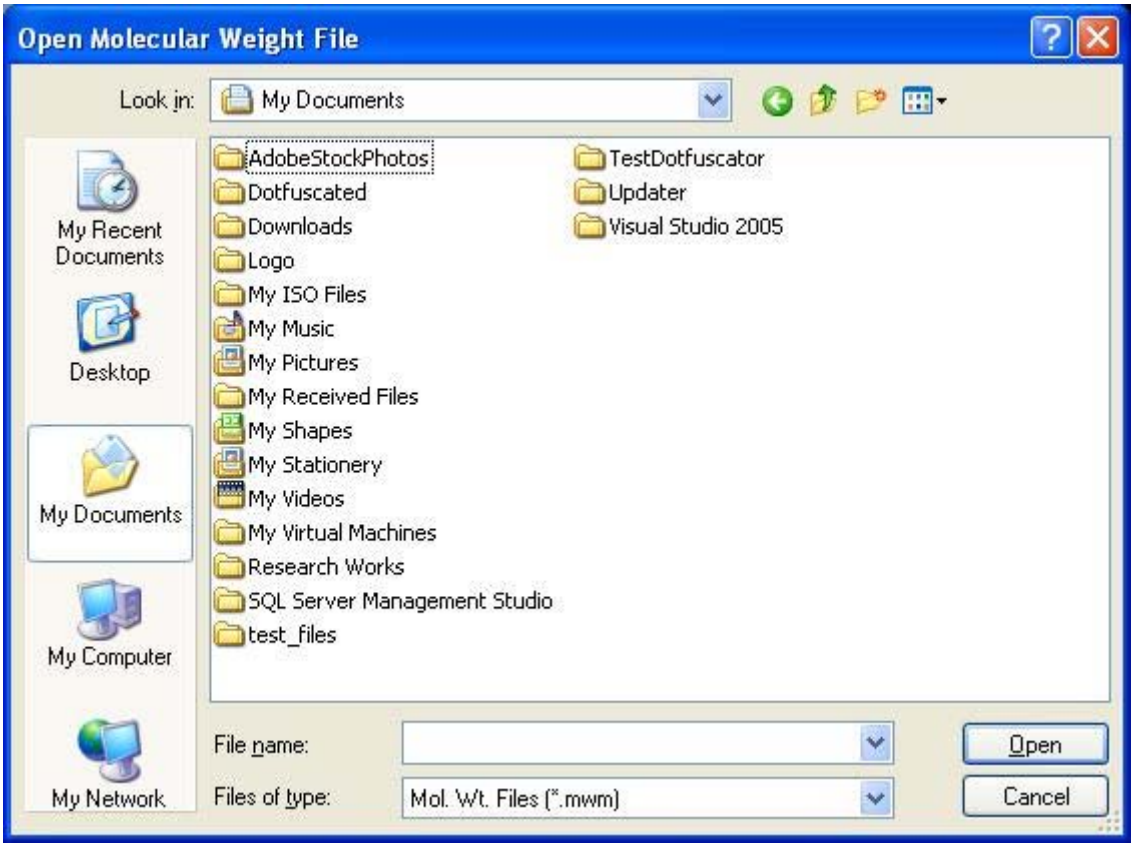
Click on the Delete Band function in the Query menu. The band will be deleted, as will the band's data in the query data table. Bands entered after the one deleted are renumbered on the image and the data table.

To delete all the queried bands, select the Clear Queries function in the Query menu.

Using the Molecular Weight Standards Library

AlphaView contains a library of DNA and protein molecular weight standards. Additional sets of markers can also be added to this library for later access. For a complete list of pre-loaded files and the band sizes in each, see Appendix B.

Applying a Set of Saved Markers
From the Marker menu in the data box, select Get Markers from File. A dialog box appears.

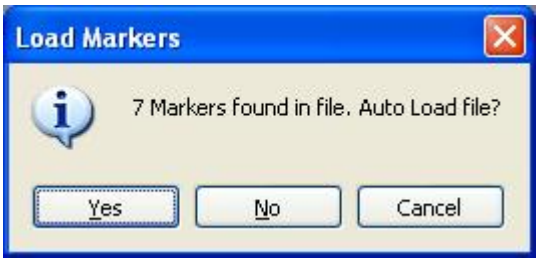


Get Markers from File Dialog Box

Using the left mouse button, click on the name of the file to be loaded. That name is then highlighted in the list and appears in the text box below the File Name: prompt.

The current directory path is shown beneath the Folders heading. Below this is a graphical depiction of the path, and the sub-directories of the current directory. If the file of interest is in a different directory than the one open, double-click on the appropriate folder icon.

Once the file has been selected, click on the OK button to load the file. (Alternatively, double-click on the file name.) The dialog box disappears and a new dialog box appears.



Auto Load Dialog Box

This box gives the file name selected, as well as the number of markers contained within the file. It also give the user the option of auto-loading the file.

If Yes is selected, a vertical line will appear attached to the cursor. Place the vertical line in the marker lane on the gel, and click. AlphaView will automatically search for the markers in the lane.

In the above example, the brightest 7 bands in the lane would be selected, and the values for Boe II's 100 bp DNA marker ladder would be assigned to the bands.

If No is selected, AlphaView will display a cursor with a red line attached. Text in the upper right-hand corner of the image window will tell the user how many markers have been entered, and the value of the next marker to be entered.

Manual Load Dialog

To begin entering markers, move the cursor to the top band in the lane and align the horizontal line with the band. Click the left mouse button. Notice that the position for that band is added to the markers data table.

Move the cursor to the next band, click the left mouse button again to assign the next value in the series. Repeat this process until the values of all the marker bands are assigned.

To quit this function before all markers have been placed, click the right mouse button.

Adding a Set of Molecular Weight Markers to the Library
If the same set of molecular weight markers will be run repeatedly, there is no need to enter values on each new image. Instead, save the molecular weight values in a file and apply them to the marker lane of any subsequent images.

To save a set of molecular weight standards, click on Write Markers to File in the Marker menu after the marker values have been entered.

Following Windows conventions (255 characters or less), enter a new file name in the text box below the File Name: prompt. AlphaView will automatically give the appropriate 3-character extension.

The current directory path is shown beneath the Directories heading. Below this is a graphical depiction of the path, and the sub-directories of the current directory. If the file is to be saved in a different directory than the one open, double-click on the appropriate folder icon.

Alternate disk drives can be accessed using the Drives heading.

Once a name has been entered and the appropriate directory has been accessed, click OK. All of the marker values that are currently in the Data window will be saved to this new file. To access them in the future, follow the steps in ***Applying a Set of Saved Markers*** above.

Special Functions:

Snap to Peak
This feature makes it easier to place the cursor on a band. If the SNAP TO PEAK function has been activated, the cursor jumps to the highest pixel value in the band as the cursor approaches it.

If bands are so tightly spaced that the line is not coinciding with the band, turn off SNAP TO PEAK and place the marker on the band manually.

If the cursor is jumping to areas between bands instead of the bands themselves, check to see that INVERT is properly set. (See below for more information.)

The INVERT Button
The INVERT function reverses the gray scale assignments so that 0 corresponds to white and 4,095 corresponds to black. If the image has dark bands and light background, then INVERT should be selected by placing an "X" in its box. (If the image has light bands and dark background, the INVERT option should not be activated.) Unlike the REVERSE button described in Chapter 3, this function does not alter the appearance of the image.

This function is especially important when using Snap to Peak. If INVERT is incorrectly set, the cursor will snap to areas between peaks rather than finding peaks.

Calculating Rf Values
To obtain accurate Rf values, specify the location of the wells (i.e., origin) and the dye front using the functions Set Well Pos./Start and Set Dye Front/End both found in the Marker menu.

To designate the location of the wells, point the cursor at Set Well Pos./Start. Position the cursor so it is pointing anywhere along the wells and click the left mouse button. A horizontal bar appears, defining the location of the origin. By clicking and dragging, the bar can be moved for better positioning.

Repeat this procedure using Set Dye Front/End to indicate the location of the dye front.

Once the origin and dye front have been defined, they are used to calculate the Rf values of any bands that are added. If dye fronts are not assigned, AlphaView uses the top and bottom of the image as the boundaries for calculating Rf values.

The Molecular Weight Cursor Box

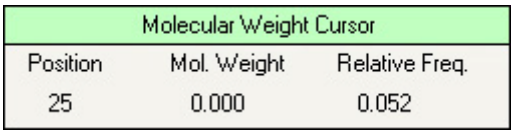


Figure 5.4 Molecular Weight Cursor Box

The MW. CURSOR box, found in the Molecular Weight toolbox, reports the location on the y-axis, the molecular weight and the R_f value of the current position of the cursor. These data are updated as the cursor is moved. This information can be helpful for positioning the cursor in a precise location, or for a quick estimate of a band's size.

The Graph Tool

The Graph function is found in the lower left corner of the Molecular Weight tools box.

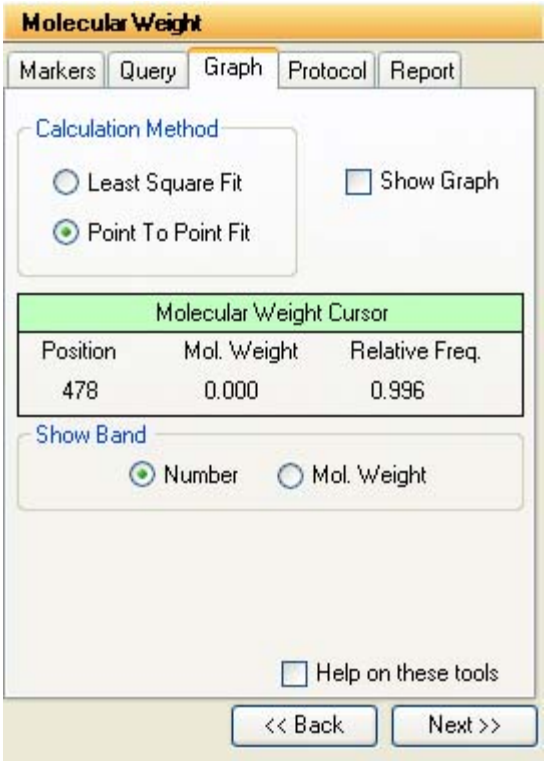
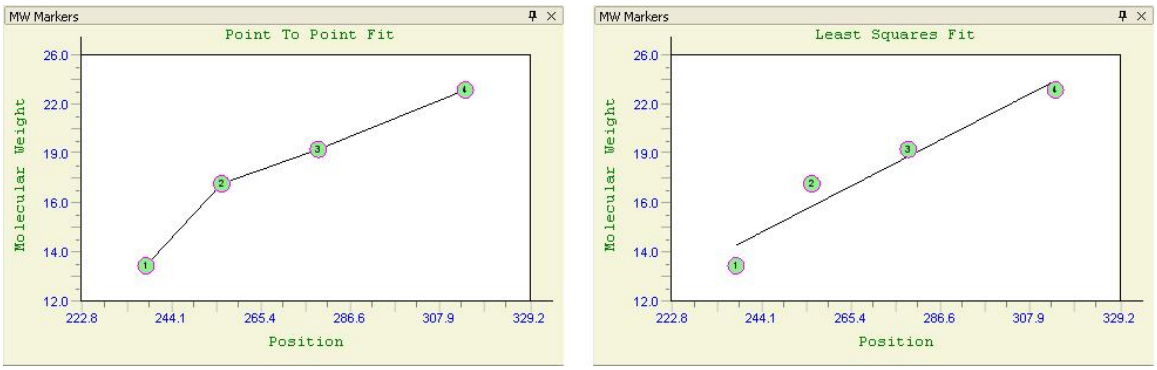


Figure 5.5 Molecular Weight Tools

Clicking in the Graph box displays the semi-log graph of the molecular weight data that is used to calculate the molecular weights of unknown bands. (To remove the graph window from the display, click on the Graph checkbox a second time.) The x-axis corresponds to the band's vertical location on the screen, and the y-axis is a log representation of molecular weight. By clicking on the appropriate button, the data can be toggled between a least squares fit and a point-to-point fit.



Example of a Point-to-Point Graph Example of a Least Squares Fit Graph

Figure 5.6 Example of Point-to-Point and Least Squares fit graph

The graph is useful to verify that the molecular weight data is entered correctly, and can help determine the most linear region of the molecular weight standards. Further, if a query band is clicked, its position will be shown on the graph by dashed lines (as shown above).

Colony Count

The Colony Count tools in the Tool Box, Analysis Tools make it easy to count the number of objects on an image, such as colonies on a Petri dish or viral plaques.

Click on the COLONY COUNT. The following sets of tools will be displayed in the lower left of the screen.

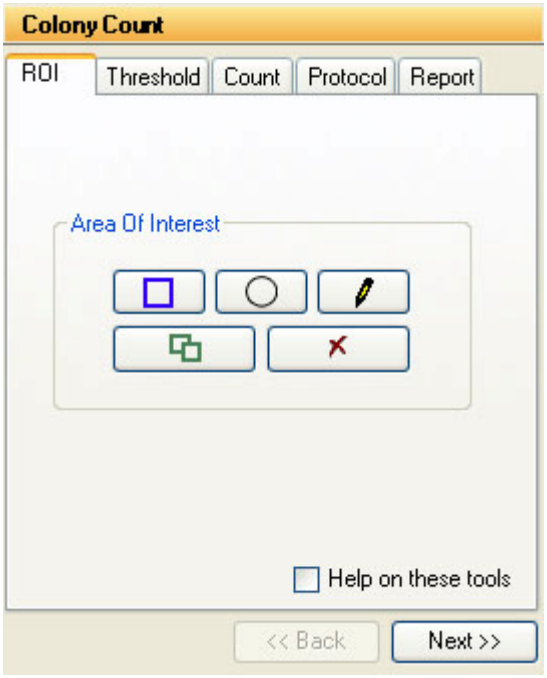


Figure 5.7 Colony Count Tools

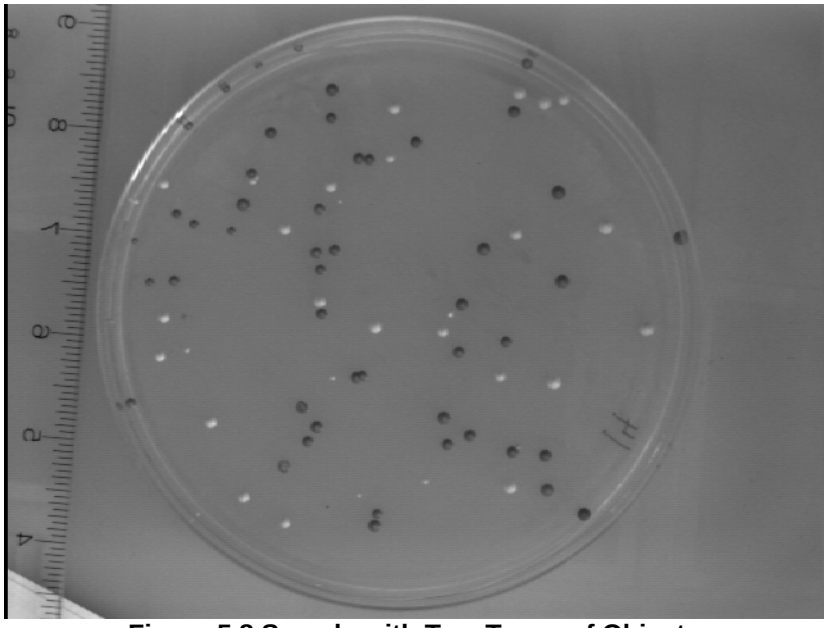


Figure 5.8 Sample with Two Types of Objects

When two types of objects are counted, the objects are highlighted in different colors. They can also be marked on the image using a "+" or an “X”.

Follow these three steps to perform an automatic colony count:

1. Define and Position Area(s) of Interest

There are three drawing tools with which areas of interest can be designated. Click on the desired AOI (Area of Interest) button in the toolbox to define areas on the image containing the objects to be counted.

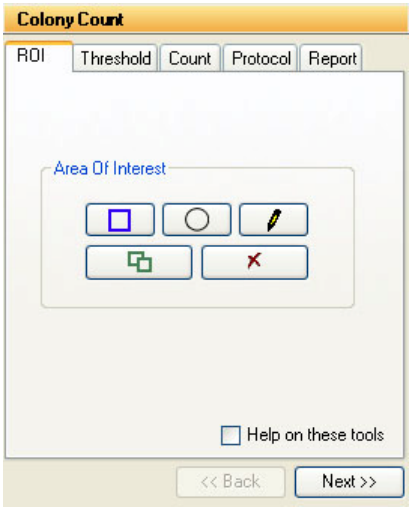
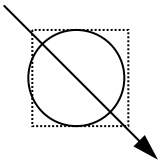


Figure 5.9 Colony Count Tools

Circles: This is the most appropriate choice for counting colonies on a Petri dish. After clicking on the button in the toolbox labeled with a circle, move the cursor into the image area to the edge of the Petri dish. Click the left mouse button and move the cursor to open the circle. When the circle corresponds to the perimeter of the Petri dish, release the mouse. The circle has “handles” around it, and can be moved or re-sized at this point.

Hint: to draw a perfect circle around a portion of an image, first visualize a square surrounding the area of interest. Position the mouse in the upper left hand corner of the square. Click and drag the mouse down across the area of interest at a 45° angle until the circle encloses the area of interest.



Rectangles: After clicking on the button in the toolbox labeled with a square, move to one of the four corners of the region to be enclosed. Click the left mouse button and move the cursor to the corner diagonally opposite. Note the box that opens as the cursor moves. Release the left mouse button when the box has reached the desired size. The rectangle has “handles” around it, and can be moved or re-sized at this point.

Freehand Drawings: To draw a freehand object, select the freehand icon; the mouse will change to a pencil. Draw an object of the desired size and shape. When the mouse is released, the start and end points of the object will be connected.

Manipulating Areas of Interest: Areas of interest can be moved, copied and deleted. To perform any of these functions, an area must first be selected by clicking on it or dragging the cursor around it. Its color changes to gray, indicating that it is active. More than one area of interest can be selected at any given time.

To move an area of interest click on the selected area. While holding down the left mouse button, drag the area to the desired location.

To delete an area of interest click on the cut button in the toolbox (designated by a pair of scissors). The selected area of interest is removed and any other areas of interest are renumbered accordingly.

To copy a selected area of interest, click on the copy button (found next to the cut button). A second copy of the area of interest appears overlapping the first one. Click and drag the copy to the desired location.

2. Set the Density Threshold(s)

The objects counted by the automatic counting routine are based on the difference in the gray levels of the objects as compared to the background (e.g., the colonies on the petri dish are either darker or lighter than the surrounding area). The density thresholds define the gray levels that are recognized by the counting routine.

To set the density threshold, click and drag the sliders. As the sliders are moved, the regions of the image that fall within the gray scale range are highlighted in the image area. Adjust the slider positions until the objects to be counted are highlighted. The numbers next to the Min and Max settings change to reflect the positions of the sliders. These numbers represent the minimum and maximum gray scale values that are currently being detected. Any object whose gray level falls between these two numbers will be counted.

If there are two sets of objects to count, click on the second colony count button (next to the COUNT button) and adjust the density threshold so the second set of objects are highlighted.

3. Count the Objects

When the density threshold settings and size settings are satisfactory, click on the COUNT button. If two types of objects are to be counted, click the second colony count button and click COUNT again. The counts are shown for each area of interest and the totals are given in an colony count display window on the image.

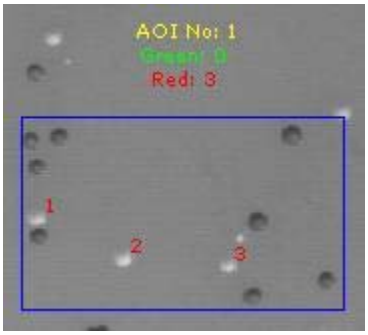
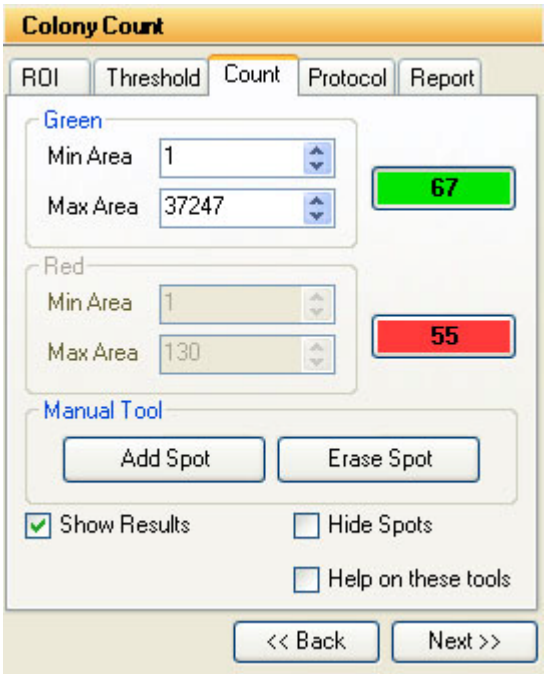


Figure 5.10 Colony Count Sample Results for an AOI

Editing Tools

After the objects have been automatically counted, AlphaView will automatically open the Edit Tools functions. If editing is necessary, these tools manually override decisions made by the automatic counter.



Colony Count Editing Tools

Displaying Spots

Spots are displayed as red (or green) numbers on the image. These can be changed to symbols ("+" or "x") by holding the <shift> key and clicking the right mouse button. Repeat to return to numbers.

Spots can be hidden from view by checking the Hide Spots checkbox.

Specify the Size Range of the Objects

If the image has objects of different sizes but only those in a certain size range are of interest, use the Area Controls.

Specify the size range by adjusting the minimum and maximum diameter settings. The numbers below the Min. Area and Max. Area headings indicate the minimum and maximum diameter settings in pixels. Any object whose diameter falls between these two numbers will be counted.

As these number are changed, objects will be added to (or deleted from) the display, and the Count windows will be updated.

Using the ADD SPOT Tool

Occasionally, the automatic counting function may fail to count an object on the image. This could occur for a variety of reasons, including inappropriate Density and Size Threshold parameter settings.

Objects can be added to the total count numbers using the Add Spot button. Click on the appropriate count box (red or green). Next, click on the Add Spot button. Point the cursor at the object to be added and click the left mouse button. The object will be highlighted and the count will automatically increase.

Figure 5.11 Results of Colony Count After Manual Addition of Three Spots

To add a second object, click on the right mouse button to re-enter "add" mode, then click on the desired object with the left mouse button.

Using the Erase Spot Tool

This function removes any extra spots that were counted either automatically or manually. For example, if a portion of an AOI has high background or other noise, a number of objects may erroneously be included in the count. Draw a box around the object or set of objects. Objects will be displayed with a white background to indicate that they are selected. Clicking Erase Spot deletes all selected objects at once and automatically reduces the total count information.

Spot Count Data

When AlphaView opens, the Colony Count data box is hidden. To display spot count data, click the checkbox to deselect Hide Data. The following data window will appear:

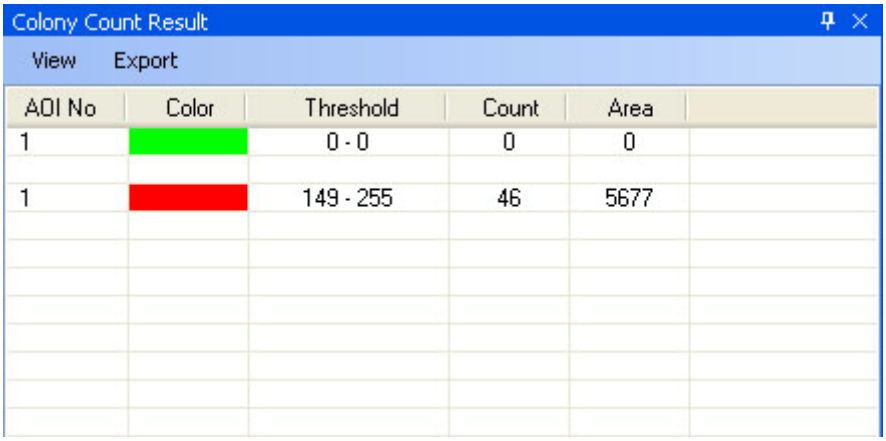


Figure 5.12 Colony Count Data Window Showing AOI Summary Data

This window shows the summary data for the area(s) of interest (AOIs). This includes the threshold values that were set for each count type, the number of objects found for each count type, and the total pixels in all of the spots.

To see specific details on each spot, select Spot Details from the View menu. The following information will appear:

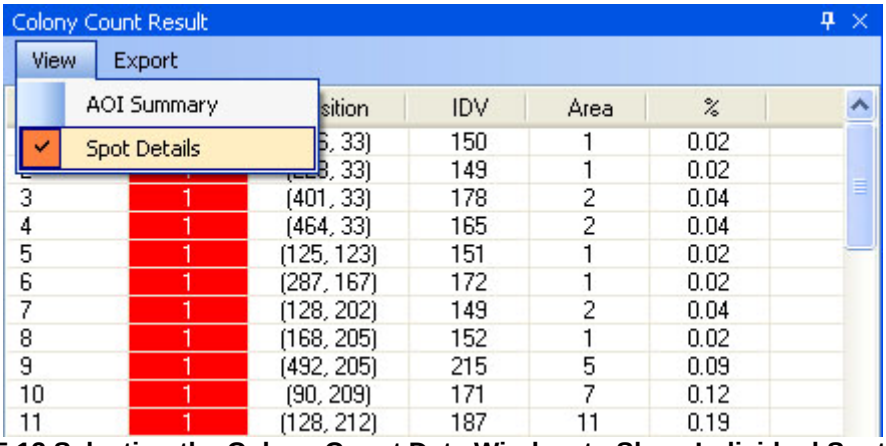
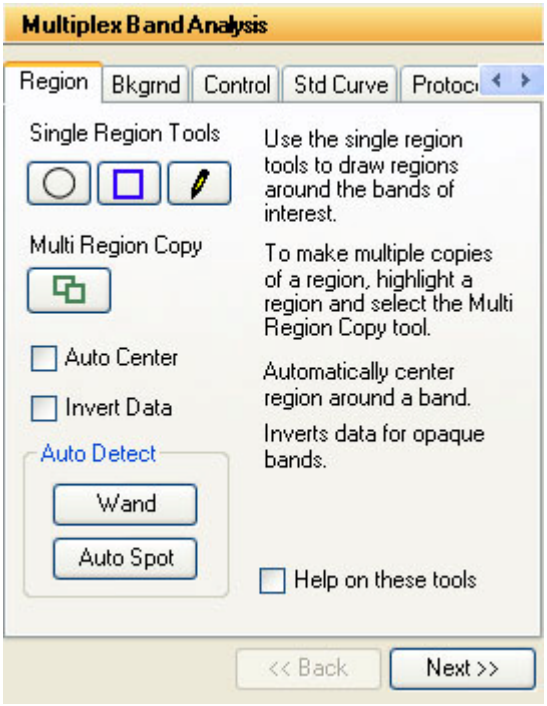


Figure 5.13 Selecting the Colony Count Data Window to Show Individual Spot Details

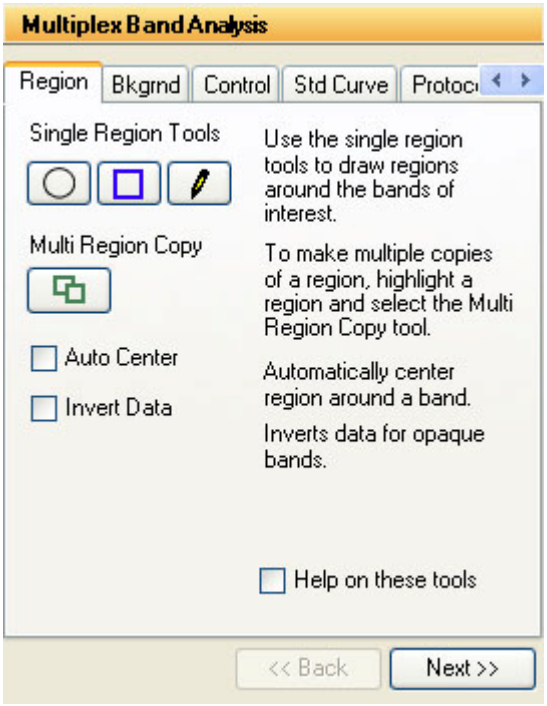
The data table gives the individual spot number, the AOI in which it was counted, its coordinates, the integrated density value (IDV) for each spot, the area (number of pixels) for each spot, and the % contribution to the total. Note: manually-added spots show areas of 1 pixel.

Multiplex Band Analysis Tools

In the ToolBox, Analysis Tools, a tab labeled Multiplex Band Analysis, opens a set of tools with which the density of bands, spots or other objects can be measured. A two dimensional area of interest (or Object) is created and the density is obtained through the corresponding pixel intensity values designated as IDV or Integrated Density Value.

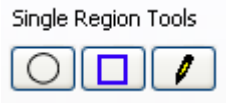


Single channel GUI



Multi channel GUI

Creating an Object Area of Interest

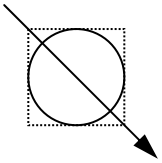
Object Boxes: 

An area of interest can be created in several ways. The user can manually draw an area of interest through the three OBJECT buttons. Objects can be enclosed with a box (rectangle), ellipse, or freehand drawing. Select the one that most closely corresponds to the shape of the objects on the image.

To draw a rectangle or ellipse, click on the button labeled with a box (or a circle) beneath the OBJECT header in the toolbox. The cursor automatically changes to a "+" when it is in the image area, indicating that AlphaView™ is in “drawing” mode. Move the "+" to the corner of the object to be measured, click the left mouse button, move the cursor, and release the mouse button when the box (or circle) surrounds the object.

Hint: to draw a perfect circle around a portion of an image, first visualize a square surrounding the area of interest. Position the mouse in the upper left hand corner of the square. Click and drag

the mouse down across the area of interest at a 45° angle until the circle encloses the area of interest.

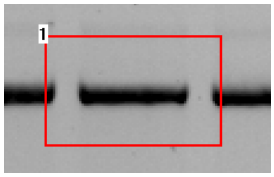


To draw a freehand object, select the freehand icon; the mouse will change to a pencil. Draw an object of the desired size and shape. When the mouse is released, the start and end points of the object will be connected.

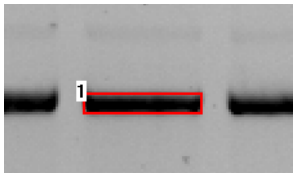
Once the mouse is released, the cursor changes to an arrow (indicating that AlphaView™ is in "edit" mode), and the object is shown in gray with handles around it. The object can now be resized using the handles or repositioned by clicking within the boundaries and dragging to a new location.

To draw another object, return AlphaView™ to "drawing" mode by clicking the right mouse button.

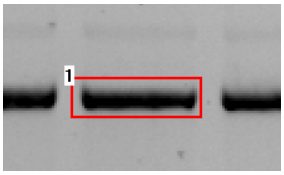
If a box or ellipse is large and includes significant background area, the corresponding density value may be large. The higher the background, the greater its contribution to the total of pixel values in that object. Therefore, objects should be drawn so that they fully enclose areas of interest, but do not include an excessive number of background pixels or pixels from neighboring regions:



Object too Large



Object too Close to Band



Recommended Object Placement

Hint: Keeping the data window open can reduce the speed at which objects are drawn and manipulated on the screen. Therefore, it may be preferable to hide the data window until all objects and backgrounds are drawn and in place.

Hint: Accurate Local Background Correction method requires the object box to contain a few pixel values that are true representations of the background level. If the object is too small and the band fills the object box then local background correction will result in significant errors.

Magic Wand and AutoSpot (Single Channel Only)

These two selections are automated detection features designed to recognize sample in an image and draw Object boxes around it. They are found under the Auto Detect heading in the main Multiplex Band Analysis. Tab. Once an object is created the dimensions can be altered and the background subtracted similar to manually drawn objects.

Figure 5.14 Magic Wand and AutoSpot Tools

Magic Wand

Selecting Magic Wand will activate a wand like symbol and the following data table:

Figure 5.15 Magic Wand Parameter Window

The tip of the wand should be centrally positioned over the sample spot on the image and then simply click the mouse. The software will use either an “edge detection” algorithm to trace an area of interest around the spot or draw a box around the sample. A right mouse click will reactivate the wand icon for another selection.

Magic Wand Sensitivity

The Magic Wand Sensitivity slide bar will alter the parameter for how much of the spot will be detected. The slide bar ranges from 0 to 100. The smaller the number the less of the spot the software will define. The greater the sensitivity number the greater the area of the spot included in data collection.

HINT: Selecting a brighter pixel within the band (or spot) will have magic wand draw a tighter area of interest. Correspondingly, a less intense pixel will draw a larger perimeter around the selected spot**

Spot Type

Under the Spot Type heading the Bright Spots selection should be checked if the image contains bright spots on a dark background (e.g. ethidium bromide stained fluorescent gels and chemiluminescent blots). The Dark Spots selection should be made when the sample contains dark sample on a light background (example: film, coomassie blue protein gels). The software should automatically determine this selection, but some manual intervention may be necessary. Remember as in all portions of AlphaView™ software if the image has been reversed (negative image) using the Reverse selection on the Contrast Adjustment window the Dark Spot selection should **not** be made. Reverse is a visual alteration only and does not affect the original image capture data. (e.g. a chemiluminescent blot is captured using the Reverse mode in order for it to appear as film, the correct selection is still Bright Spots).

Outline Type

Border Outline will use the edge detection methods to determine the spot area. The Box Outline will draw a box shape around the sample usually including a significant portion of background. User preference and image particulars will determine which outline type is best.

Previous Location

Previous Location will automatically select the spot on the image last selected for magic wand and apply any changes made for sensitivity. This feature is designed to quickly evaluate how the different sensitivity numbers will affect the drawn area using the same selected point in a spot. The X,Y coordinates are given for the previous spot as well.

Exit

Exit will remove the Magic Wand Parameter Window.

Auto Spot

Auto Spot is a feature designed to find multiple spots within a region of interest. It is accessed through AutoSpot under Auto Detect in the Multiplex Band Analysis tab. Once it is selected the following tab will appear and the cursor will be ready to draw an area of interest.

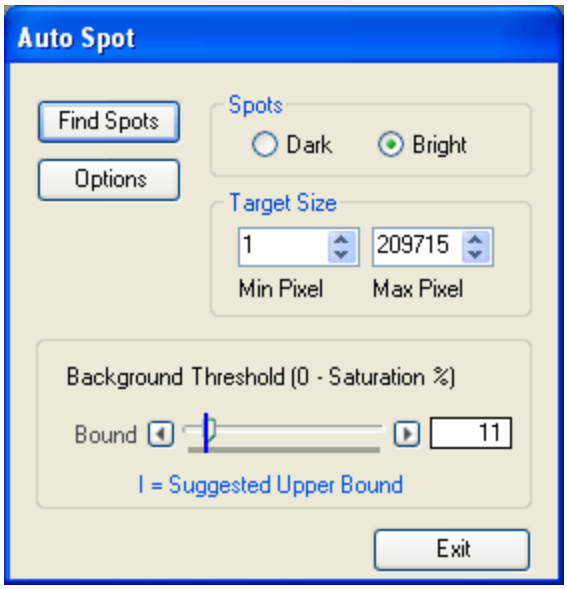


Figure 5.16 Auto Spot

Area of Interest

After selecting AutoSpot an area should be drawn on the image by clicking and dragging the mouse. The smaller and more defined the Area of Interest is, the better the resulting data. The area can be manipulated using the manipulation tabs at the edge of the red box.

Options

The Options selection allows either a box outline to be drawn around the spots or a border outline. The box will include more of a background in the drawn area than the border outline. User preference and image particulars will determine which outline type is best. The sensitivity can also be adjusted by sliding the bars for normal spots (<98% saturation) and for saturated spots (>98% saturation).

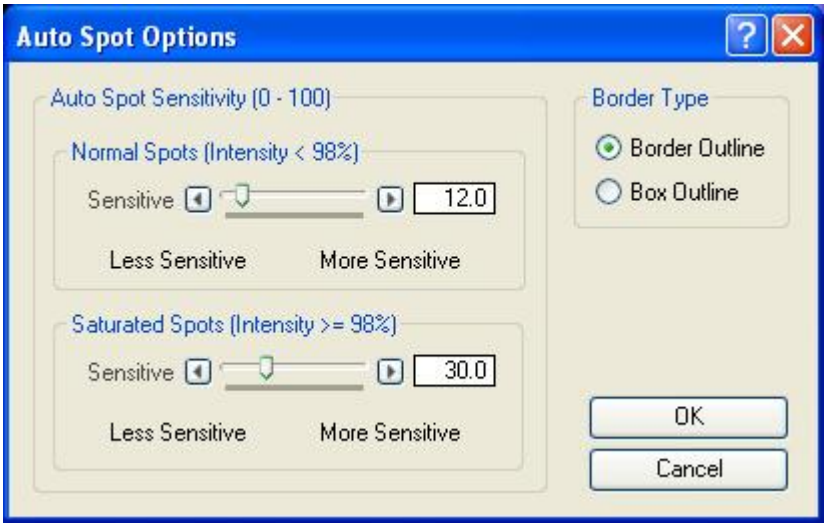


Figure 5.17 Auto Spot Options

Bright Spot vs Dark Spot

The Bright Spots selection should be checked if the image contains bright spots on a dark background (e.g. ethidium bromide stained fluorescent gels and chemiluminescent blots). The Dark Spots selection should be made when the sample contains dark sample on a light background (film, commassie blue protein gels etc..). The software should automatically determine this selection, but the user may have to override it if the software incorrectly evaluates an image. Remember as in all portions of AlphaView™ software if the image has been reversed (negative image)using the Reverse selection on the Contrast Adjustment window the Dark Spot selection should not be made. Reverse is a visual alteration only and does not effect the original image capture data. (EG if a chemiluminescent blot is captured using the Reverse mode in order for it to appear as film, the correct selection is still Bright Spots).

Find Spots

The Find Spot button is selected once the correct area of interest is drawn and the Bright Spot/Dark Spot selection is correctly made. A green outline will be drawn around the detected spots.

Background Threshold

The background threshold slide bar will adjust the criteria used for finding spots. It is on a scale of 0 to 100% saturation. The sliding bar maps the dynamic range of the camera. By calculating the fringe pixels in the image or ROI, the initial threshold value and suggested lower (or upper) bound value are calculated and shown on the sliding bar. The user can always override the threshold value by moving the sliding bar or typing in the counter box.

Target Size

The user can input the minimum and maximum area in pixels of the spots or bands of the desired targets. The default values are 1 pixel minimum and no limit for maximum pixels.

Get Data

Get Data is selected once a satisfactory spot outline is achieved. This will convert all drawn objects into standard Spot Densitometry objects with associated density numbers.

Manipulating Objects

Selecting Objects

To select an object, click on it with the mouse. "Handles" will appear at its corners.

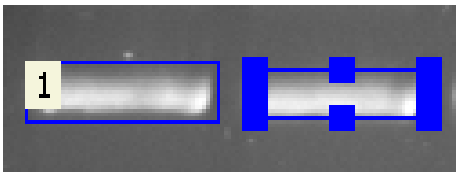


Figure 5.18 Non-Selected and Selected Objects

To select a second object ***instead of*** the first, click on the desired object.

To select more than one object at a time, drag the mouse around all of the objects of interest. Note: an object must be ***completely*** surrounded by the mouse operation in order to be selected.

To select more than one object where it is not feasible to drag the mouse around objects, hold the `<shift>` key and click on each object to be selected.

To de-select an object, click the left mouse button outside of the selected object. The handles disappear, indicating that the object is no longer selected.

Copying Objects

To draw boxes or ellipses enclosing the same number of data points, use the COPY function.

First, draw or select an object of the desired shape and size as described above. Next, click on the COPY button in the toolbox. A duplicate of the selected object will appear on the screen. This object can then be moved to the desired position.

To make multiple copies of an object, continue to click on the COPY button until the desired number of copies is displayed.

Moving Objects

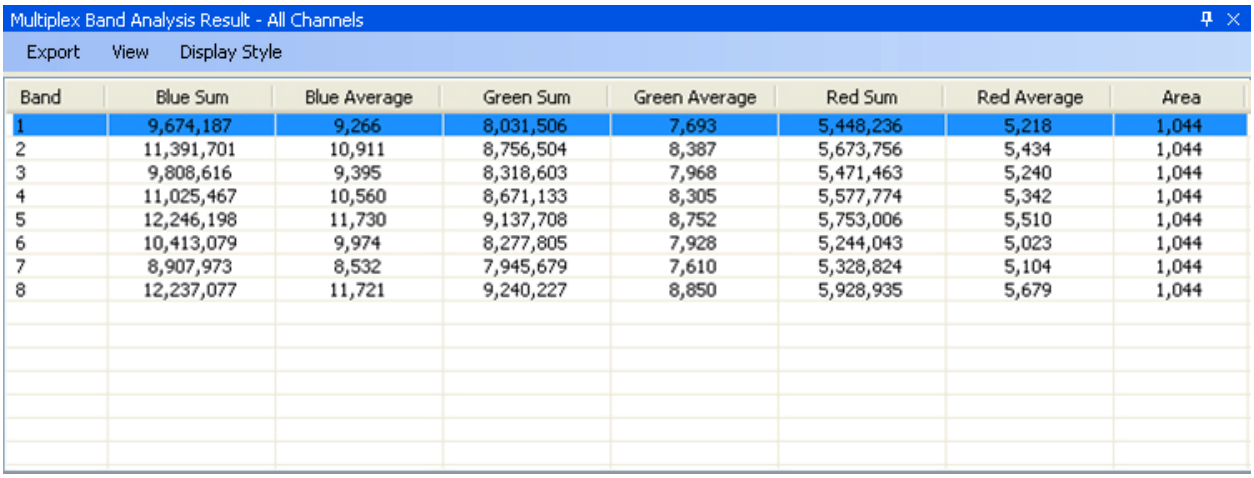
An object can be moved to fine-tune its position simply by clicking on it, holding down the left mouse button and dragging it to the desired location.

Deleting Objects

To remove an object or set of objects, select the object(s). Select DELETE button on keyboard. All the selected objects are removed from the image window and their associated data is cleared from the data window.

Multiplex Band Analysis Measurements

As objects are drawn, their density data is automatically calculated and displayed in a data window. Any time an object is drawn or detected, the data in the window are updated. If the Multiplex Band Analysis Results window obscures a region of interest, resize and/or move it following Windows® conventions.



Band	Blue Sum	Blue Average	Green Sum	Green Average	Red Sum	Red Average	Area
1	9,674,187	9,266	8,031,506	7,693	5,448,236	5,218	1,044
2	11,391,701	10,911	8,756,504	8,387	5,673,756	5,434	1,044
3	9,808,616	9,395	8,318,603	7,968	5,471,463	5,240	1,044
4	11,025,467	10,560	8,671,133	8,305	5,577,774	5,342	1,044
5	12,246,198	11,730	9,137,708	8,752	5,753,006	5,510	1,044
6	10,413,079	9,974	8,277,805	7,928	5,244,043	5,023	1,044
7	8,907,973	8,532	7,945,679	7,610	5,328,824	5,104	1,044
8	12,237,077	11,721	9,240,227	8,850	5,928,935	5,679	1,044

Figure 5.19 Example of a Multiplex Band Analysis Data Window

Data Definitions

See appendix D for a complete listing and description of the various quantities. The basic set of measured quantities are sum, average, percentage, area, position and standard deviation. These values are calculated for all regions in all color channels including the background regions.

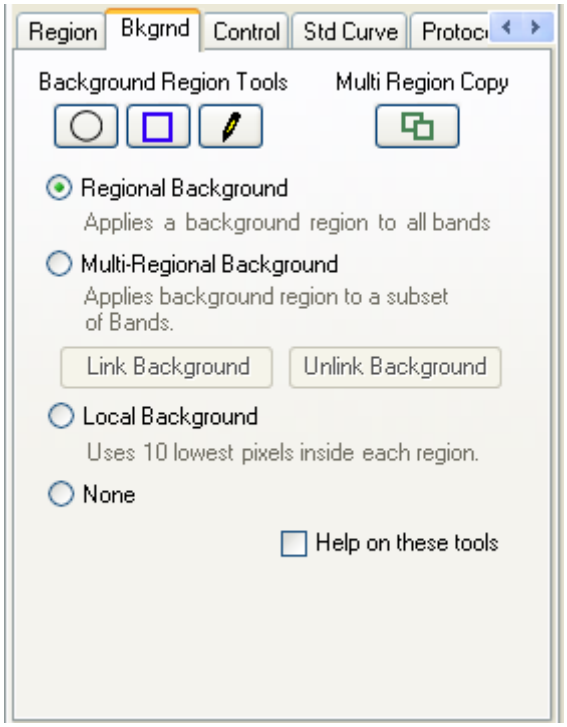
Inverting Data

The INVERT function reverses the gray scale assignments so that 0 corresponds to white and 65,535 corresponds to black. This control is found on the REGION tab.

If the image has dark bands and light background, then INVERT should be selected by placing an "X" in its box. If the image has light bands and dark background, the INVERT option should not be activated.

Note: This function does not alter the appearance of the image (unlike the REVERSE button described in Chapter 2).

Background Tab – Calculating background values



Background tab

Background subtraction is an important part of image densitometry analysis. The **Background** tab is used to subtract the background in the image from the regions of interest. The background is unwanted signal in the region arising from the fluorescence detection chemistry, the membrane and quality of the sample itself. Because fluorescence detection is extremely sensitive, high background levels in the image can be a common problem, especially in the early stages of protocol development. Fluorescence protocols require careful attention to cleanliness and sample handling to minimize background problems.

Regional Background

Background region applied to all object regions. Place a background region in an area of the image representative of the background level for all channels. The image contrast display should be adjusted to so that the variations in the background can be seen so that the background region(s) may be placed in an area best representative of the background. Note that more than one background region can be used and the average pixel level of all the background regions is applied. The data table is automatically updated with Background Corrected (BC) values.

Multi- Regional Background

Background region applied to a subset of object regions. A second (or third) background region can then be applied to a second (or third) subset of object regions. Each subset of object regions is corrected by the linked background region to account for differences in background level across an image.

Local Background

Background region applied using the average of the 10 lowest pixels in a band region. The regions should be slightly larger than the bands to use properly apply this method of background correction. The data table is automatically updated with the Background corrected intensity values.

None (No Background)

If no background calculation method has been selected, the background value in the data window (BACK) is reported as zero (0). Therefore, the object's integrated density value will be equal to the sum of all the pixel values within the box, ellipse, or freehand drawing. ***Recommendation:*** No Background or Manual Background is suggested for AutoSpot, Magic Wand (only the border outlines though) or any object where little if any background is included in the object area of interest.

Background Link/Unlink Tool

Link Background

Applied in conjunction with Multi-Regional Background to link a background region to a subset of object regions. First place a background region in an area of the image representative of the background specific to the subset of object regions. Draw a rectangle by left clicking on the image and dragging to include the desired subset of bands and a single background region to be linked. Alternatively use Ctrl left click to select multiple regions and the specific background region. Then select the **Link Background** button. The data table is updated with Background corrected values.

Unlink Background tool

Used to unlink the background regions from the band subsets.

Figure 5.20 Unlink Background Tool

B1 (background) and data box 2 (region) are selected and Linked as shown.

When using the region and multiregional background correction methods there are two points to consider. First, note that a background region applied to a multichannel image extracts and applies the background values on each channel using the respective background and region values for that channel. For a three channel image, for example, Blue, Green and Red background values are extracted and applied to the Blue, Green Red channel object regions respectively. Second, note that the background correction is automatically adjusted for any differences in area between the background and object regions as f006Fllows.

Data Definitions

Background Corrected (BC) Average:	Background Corrected Average = (Region Average – Bkgd Region Average)
Background Corrected (BC) Sum:	Background Corrected Sum = (BC region Average *Region Area)

For regions with different area the relevant quantities are adjusted for the relative region area to obtain accurate corrected values. For example if local background correction is used the "area" is 10 pixels for the background region. When this value is used to correct a data region the actual value used is scaled such that the 10 pixel area appears to be the same area as the data region. In this way regions with different areas are accurately accounted for in all calculations.

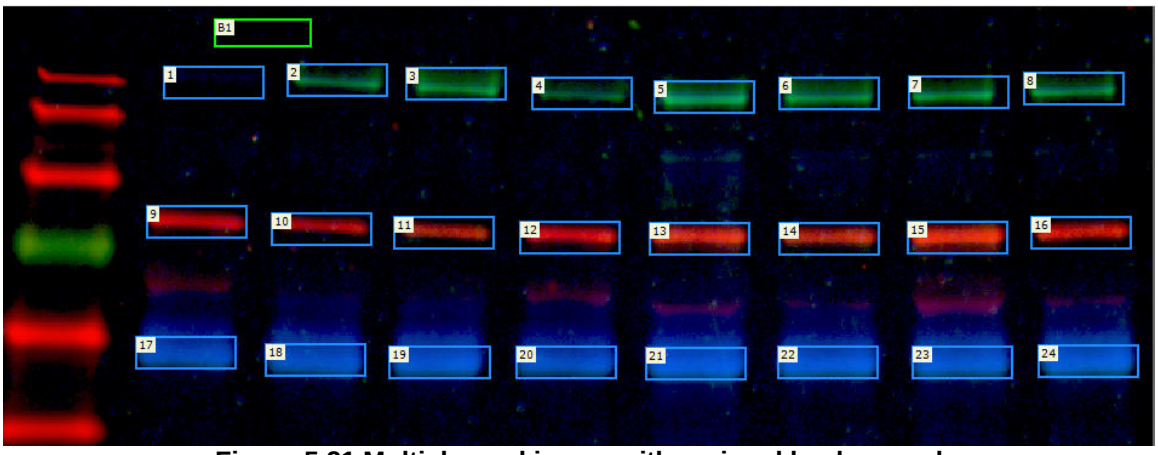


Figure 5.21 Multichannel image with regional background

A multichannel image with regional background applied. The background values from a single region are extracted and applied to each of the three channels respectively.

Multiplex Band Analysis Result - All Channels

Export View Display Style

Band	Blue BC Average	Blue Signal/Noise	Green BC Average	Green Signal/Noise	Red BC Average	Red Signal/Noise
1	45	0.84	-104	-0.83	23	0.50
2	74	1.38	841	6.69	27	0.58
3	77	1.44	1,558	12.39	29	0.62
4	74	1.38	566	4.50	22	0.47
5	153	2.86	2,233	17.76	19	0.41
6	102	1.90	1,617	12.86	15	0.32
7	67	1.25	1,454	11.57	4	0.09
8	62	1.16	1,232	9.80	8	0.17
9	28	0.52	-5	-0.04	2,094	45.13
10	39	0.73	94	0.75	1,335	28.77
11	39	0.73	216	1.72	1,247	26.87
12	59	1.10	135	1.07	2,324	50.08
13	67	1.25	352	2.80	3,061	65.96
14	55	1.03	328	2.61	1,969	42.43
15	52	0.97	371	2.95	3,689	79.50
16	20	0.37	218	1.73	2,199	47.39
17	1,315	24.55	916	7.29	141	3.04
18	1,508	28.15	1,090	8.67	55	1.19
19	1,536	28.67	1,140	9.07	43	0.93
20	1,408	26.28	1,002	7.97	75	1.62
21	2,005	37.43	1,449	11.53	102	2.20
22	1,810	33.79	1,381	10.99	62	1.34
23	1,671	31.19	1,358	10.80	152	3.28
24	1,702	31.77	1,417	11.27	70	1.51

Figure 5.22 Band Correct Values

The data table showing the respective Background Corrected (BC) Average values for each channel after regional background correction. Note that the Signal/Noise ratio is greater than 3 (indicating a significant signal level) for regions 17 to 24 in the Blue channel, regions 2-8 and regions 17-24 in the Green channel and for regions 9 to 16 in the Red channel corresponding to the visible bands in the image. Be aware that the actual biological variation (best seen by observing the signals from replicate samples) will be much greater than the signal/noise ratios from the image data.

Control Tab

Control tab is used to apply loading controls and a positive control to the data. Note that while the background regions are not displayed in the control tab the background corrected data values are maintained in the Data Table and are used in the calculations.

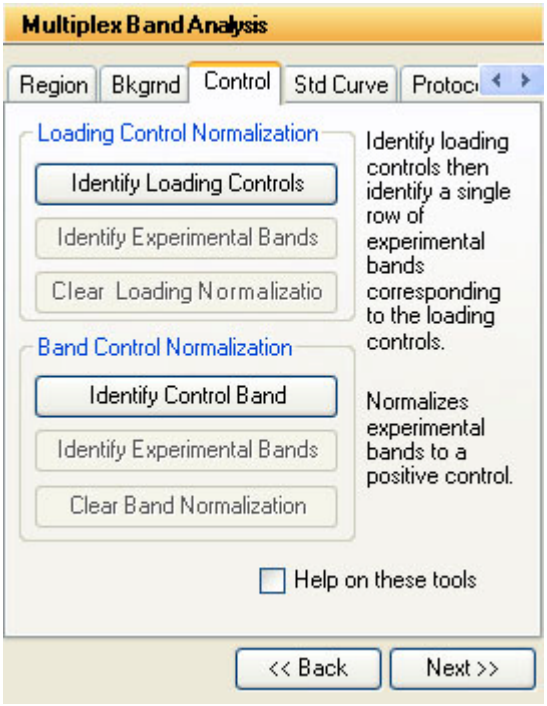


Figure 5.23 Control Normalization Tab

Loading Control Normalization Tools

Normalize experimental bands to the corresponding loading controls to adjust data for variations in the amount of sample loaded in each lane. The loading controls may be in a different color channel than the experimental bands in a multicolor image. In this case the loading control protein is labeled with Dye 1 (blue) and the experimental protein is labeled with another dye (Dye 2). To perform loading control normalization, first identify the row of loading controls, and then identify a row of experimental bands. A second (and third) row of experimental bands may then be identified by repeating this process.

Identify Loading Controls

Select the channel for loading control normalization. Identify each member of the row of loading control bands using the mouse pointer. You may either left click on each of the regions individually or draw a rectangle to select a single row of regions. Deselect the **Identify loading controls** button when done (or right click to deselect tool). All loading controls must from the same color channel as would be expected for a protein represented in each lane identified with the same dye (color).

Identify Experimental Bands

Identify each member of a single row of experimental bands using the mouse pointer. You can either left click on each of the region or draw a rectangle to select a row of regions. The **Identify Experimental Bands** button will automatically deselect when the number of bands identified in the row reaches the number of loading control bands.

There may be multiple rows of experimental bands in the same or different channels corresponding to other proteins labeled with the same or different fluorophores.

Each row of experimental bands must have the same number of regions as the loading control row so that the correspondence between the loading controls and experimental bands can be established. If the signal for an experimental band is absent in one lane of a row, place a region in the corresponding lane where the signal is expected.

The software identifies the correspondence between the loading controls and experimental bands on the basis of their positions in the image. The leftmost loading control is matched with leftmost experimental band. The order in which bands are selected does not matter. The lane correspondence for the loading controls and experimental bands is indicated by the matching colors of the regions.

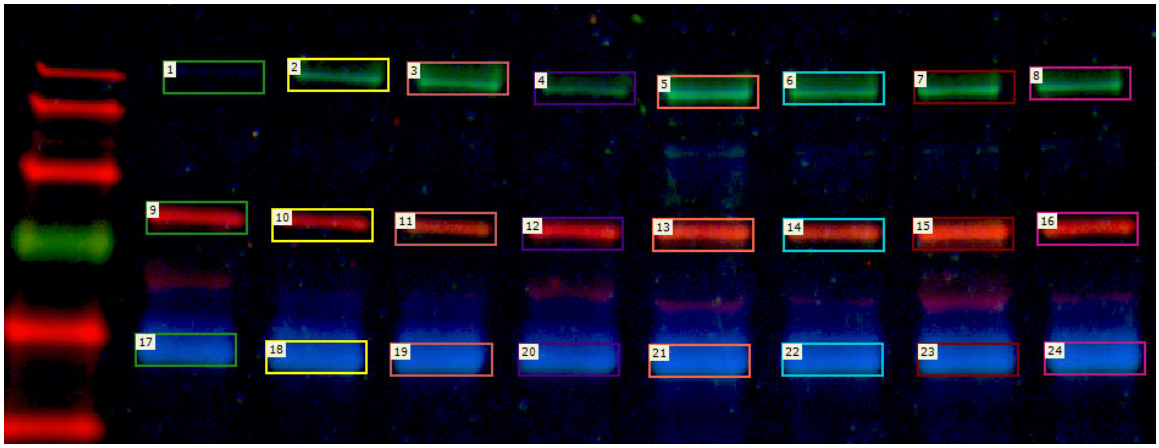


Figure 5.24 Loading Control Normalization

In this example the Blue channel regions 17 to 24 were identified as the loading controls. The regions 1 to 8 were identified as one set of experimental proteins and the regions 9 to 16 were identified as another set of experimental proteins. Note that region 1 was created even though no Green signal level is present so that there are equal numbers of Green and Red bands for loading control normalization.

The lane membership of each region is color coded for ease of identification. Region 18 is the loading control for regions 2 and 10 (all color coded yellow). Notice in the above example that all the regions in the same vertical lane are color coded with the same color.

Multiplex Band Analysis Result - Maximum Intensity Channel

Export View Display Style

Band	Channel	Sum	Average	BC Sum	BC Average	LCN Sum	LCN Average	LC Regions
1	Blue	1,006,046	882	51,300	45	-146,002	-128	Green 1 / Blue 17
2	Green	2,749,126	2,411	958,740	841	1,029,549	903	Green 2 / Blue 18
3	Green	3,566,145	3,128	1,776,120	1,558	1,872,529	1,643	Green 3 / Blue 19
4	Green	2,435,645	2,136	645,240	566	742,106	651	Green 4 / Blue 20
5	Green	4,335,657	3,803	2,545,620	2,233	2,056,017	1,804	Green 5 / Blue 21
6	Green	3,633,284	3,187	1,843,380	1,617	1,649,239	1,447	Green 6 / Blue 22
7	Green	3,448,302	3,024	1,657,560	1,454	1,606,350	1,409	Green 7 / Blue 23
8	Green	3,195,339	2,802	1,404,480	1,232	1,336,298	1,172	Green 8 / Blue 24
9	Red	3,022,677	2,651	2,387,160	2,094	2,939,701	2,579	Red 9 / Blue 17
10	Red	2,156,907	1,892	1,521,900	1,335	1,634,302	1,434	Red 10 / Blue 18
11	Red	2,056,935	1,804	1,421,580	1,247	1,498,744	1,315	Red 11 / Blue 19
12	Red	3,284,515	2,881	2,649,360	2,324	3,047,093	2,673	Red 12 / Blue 20
13	Red	4,125,500	3,618	3,489,540	3,061	2,818,391	2,472	Red 13 / Blue 21
14	Red	2,880,273	2,526	2,244,660	1,969	2,008,258	1,762	Red 14 / Blue 22
15	Red	4,840,962	4,246	4,205,460	3,689	4,075,534	3,575	Red 15 / Blue 23
16	Red	3,142,296	2,756	2,506,860	2,199	2,385,162	2,092	Red 16 / Blue 24
17	Blue	2,454,023	2,152	1,499,100	1,315	-	-	-
18	Blue	2,673,698	2,345	1,719,120	1,508	-	-	-
19	Blue	2,706,068	2,373	1,751,040	1,536	-	-	-
20	Blue	2,560,063	2,245	1,605,120	1,408	-	-	-
21	Blue	3,240,541	2,842	2,285,700	2,005	-	-	-
22	Blue	3,018,247	2,647	2,063,400	1,810	-	-	-
23	Blue	2,859,167	2,508	1,904,940	1,671	-	-	-
24	Blue	2,895,561	2,539	1,940,280	1,702	-	-	-

Figure 5.25 Loading Control Normalization Data Table

The Loading Control Normalized values (LCN) are displayed in the Max channel format.

By multiplying the normalized values by the mean of all the loading controls, the normalized values are scaled so that the LCN Average and LCN Sums can be compared to the BC Average and BC Sum to see the effect of normalizing to the loading controls.

Clear Loading Normalization
Function clears all normalization currently active on the image.

Data Definitions

LCN Sum — Loading Control Normalized Sum = (LCN Average* Area)

LCN Average — Loading Control Normalized Average = (BC Average of Experimental Band/BC Average of corresponding Loading control) * mean of all loading controls

LC Regions — Specifies the channel and region number used for each Experimental Band and corresponding Loading Control, (e.g. Red1/ Green 12)

Band Control Normalization

Normalize regions of experimental bands to a reference band control. First select the channel and identify the region for the positive control and then identify the regions for the experimental bands. The control band and the corresponding experimental bands must be in the same channel. Multiple groups of positive controls and experimental bands may be identified and each set may be in a distinct channel.

Identify Control Band
Identify the control band using the mouse pointer. Select the Identify **Experimental Bands** tool to identify the experimental bands to be normalized to this control band. Bands can be selected

You can either left click on each of the regions or draw a rectangle to select a row of regions. Deselect the Identify Experimental Bands or right click when done.

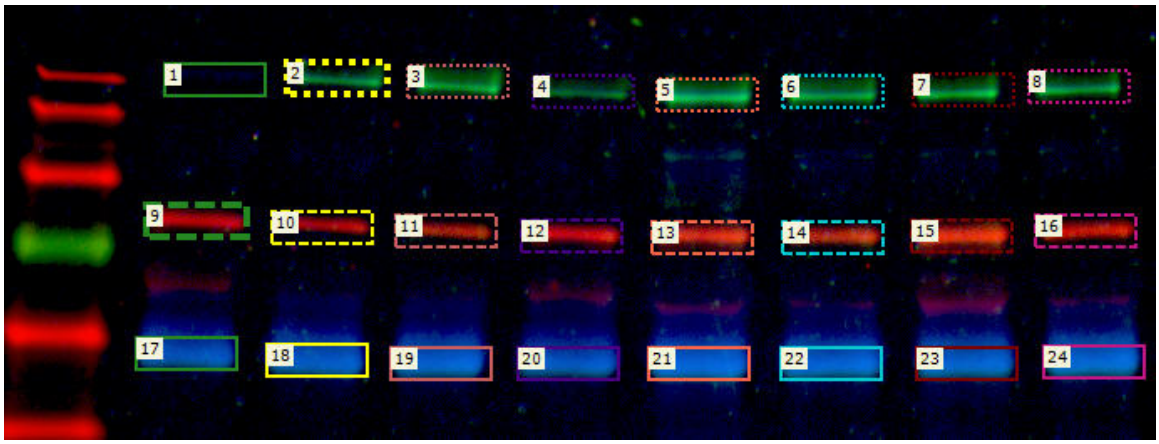


Figure 5.26 Band control normalization

In this example, region 2 in the Green channel was selected as a control band and regions 3 to 8 were selected as the associated experimental bands. Then region 9 was selected as a control band in the Red channel and regions 10-16 were selected as the associated experimental bands. Note that the regions outlines are dashed to indicate the corresponding control and experimental bands.

After identifying the control and experimental bands the Data table is updated with four additional columns, PCN Sum, PCN Average, PC Regions and Fold Change (PCN Positive Control Normalized). The control band is assigned a PCN Average value of 100 and the normalized experimental bands are adjusted to this value in PCN average column.

Multiplex Band Analysis Result - Maximum Intensity Channel

Export View Display Style

Band	Channel	LCN Average	NCN Sum	NCN Average	NC Regions	Fold Change
1	Blue	-128	-	-	-	-
2	Green	903	-	100.00	-	±1.00
3	Green	1,643	207,342	181.88	Green 3 / Green 2	+1.82
4	Green	651	82,172	72.08	Green 4 / Green 2	-1.39
5	Green	1,804	227,659	199.70	Green 5 / Green 2	+2.00
6	Green	1,447	182,617	160.19	Green 6 / Green 2	+1.60
7	Green	1,409	177,868	156.02	Green 7 / Green 2	+1.56
8	Green	1,172	147,966	129.79	Green 8 / Green 2	+1.30
9	Red	2,579	-	100.00	-	±1.00
10	Red	1,434	63,377	55.59	Red 10 / Red 9	-1.80
11	Red	1,315	58,120	50.98	Red 11 / Red 9	-1.96
12	Red	2,673	118,165	103.65	Red 12 / Red 9	+1.04
13	Red	2,472	109,296	95.87	Red 13 / Red 9	-1.04
14	Red	1,762	77,879	68.32	Red 14 / Red 9	-1.46
15	Red	3,575	158,047	138.64	Red 15 / Red 9	+1.39
16	Red	2,092	92,495	81.14	Red 16 / Red 9	-1.23
17	Blue	-	-	-	-	-
18	Blue	-	-	-	-	-
19	Blue	-	-	-	-	-
20	Blue	-	-	-	-	-
21	Blue	-	-	-	-	-
22	Blue	-	-	-	-	-
23	Blue	-	-	-	-	-
24	Blue	-	-	-	-	-

Figure 5.27 Band control normalization data table

In this example, region 2 in the Green channel corresponds to a negative control band and Region 9 in the Red channel corresponds to a negative control band. The PCN average values for regions 3 to 8 show the signal level relative to the control band and regions 10 to 16 show the signal level relative to the control band.

If a loading control has been applied to all selected bands then the loading control normalized LCN average values are used in the PCN calculation. If loading control normalization has not been done then the BC values are used and if no background correction has been applied then the Average values are used.

Data Definitions

PCN Sum Positive Control Normalized Sum= (PCN Average *Area)

PCN Average Positive Control Normalized Average = (LCN Average of experimental band / LCN Average of control band) *100.

PC Regions Specifies the channel and region number used for each Experimental Band and corresponding Band Control, (e.g. Red1/ Red 6)

Fold Change Fold Change of experimental band relative to control
If LCN Average experimental band > LCN Average control band then fold change = LCN average experimental / LCN average control If LCN Average of the experimental band < LCN Average of control then fold change = (-1) * 1/ (LCN average experimental / LCN average

Note: In the Data table there are columns available for Blue/Green, Blue/Red, etc. These columns display the ratio of the indicated channel Sums (or BC Sums) for that region and are intended for analysis of dual labeled bands or spots as may encountered in dot blots. These ratios are somewhat arbitrary as they depend on the relative exposure times of the channels. Do not confuse these ratios with the LC regions or BC regions which indicate which regions are selected as control and experimental bands respectively.

See Appendix X for a detailed example of analysis of a phosphorylation study using multicolor western methods using loading controls and Band controls.

Mass Standard Calibration Curves for Quantitative PCR

The button in the Multiplex Band Analysis toolbox labeled STD CURVE opens a set of tools that create a calibration curve for applications such as quantitative PCR and Western Blot band quantization. The calibration curve functions allow quantization of the bands on a gel based on a set of standards. A minimum of two standard bands must be input, but the accuracy of the calibration curve increases as the number of standard bands and their range of values increase.

Defining the Bands as Objects

Before generating a standard curve, define the bands using the object drawing tools in the Multiplex Band Analysis toolbox. As described in ***Drawing Objects*** above, the size of an object can influence the integrated density value. Therefore, we recommend using the same sized box, ellipse or freehand drawing when defining a standard curve. (See ***Copying Objects*** above for instructions.)

Specifying the Quantization Units

Once the objects are defined, click on the STD CURVE button. The Standard Curve Toolbox will appear. Enter the units in which the results should be reported (e.g. ng, ul, pg, %) in the **Enter units** box

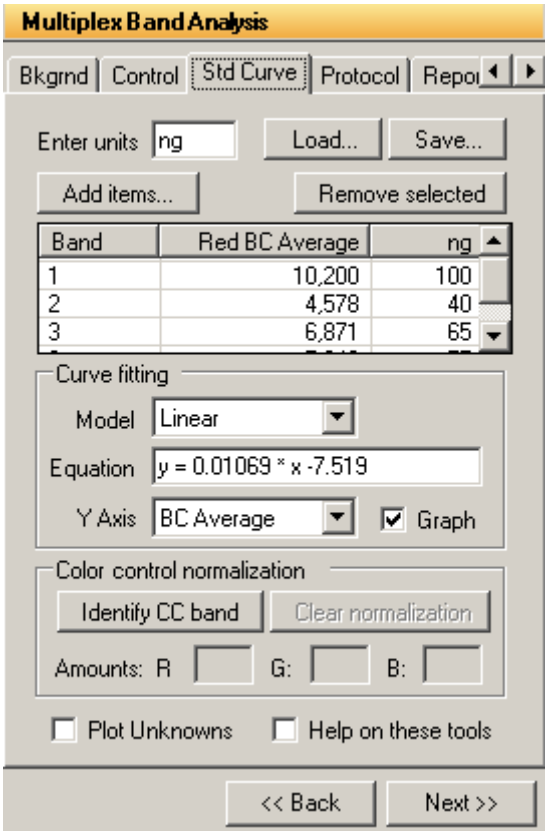


Figure 5.28 The Standard Curve Toolbox

Designating the Standard Bands

Select **Add Items** and select the color channel (in the above figure the Red channel has been selected) and select the appropriate regions created using the object drawing tool. Continue to select regions until all regions used for standard curve calculation are listed. For each band whose value (in the above figure Red background corrected average is selected) is known enter the concentration in the last column as shown in Figure 5.40

Band	Red BC Average	ng
1	10,200	100
2	4,578	40
3	6,871	65

Figure 5.29 The Standard Curve input for known concentration

(expressed in nanograms in example above)

In the Band/Sum/concentration box displayed, enter the value for the band. When the value is inserted, click on the **OK** button. The band number changes from white to green, indicating that it is now a standard (Figure 5.41).

Band	Red BC Average	Red ng
1 s	10,200	100.00
2 s	4,578	40.00
3 s	6,871	65.00
4	7,020	67.55
5	1,345	-
6 s	7,348	75.00

Figure 5.30 The Standard Curve spreadsheet

Once a second value has been entered, a curve is displayed. The points corresponding to the standard bands are labeled in green. Points for the unknown objects on the image are displayed on the standard curve based on their selected values. These are labeled in white. Enter values for each band whose amount is known. As more standard points are added, the calculated values of the unknown points may change. The relevant values for the best fit curve and the corresponding equation, along with the coefficient of correlation, is displayed.

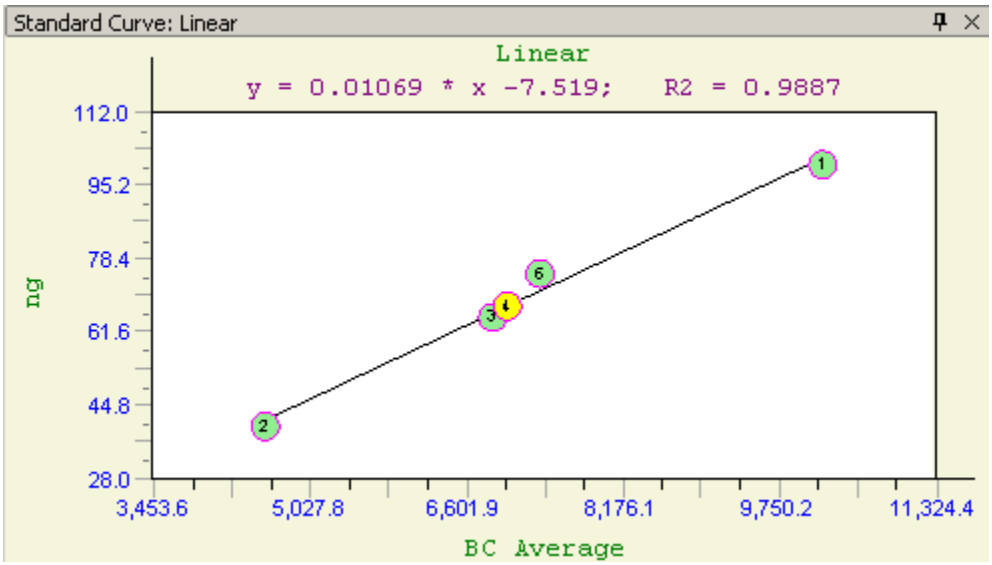


Figure 5.31 Standard Curve

(Green spots are the entered standard values; Yellow spots are quantified unknown values)

NOTE: Cubic spline and point-to-point curves have no equation nor r^2 value. These values are displayed only for linear regression and log linear regression fit models.

To remove a band from the set of standards, click on it again. The band number changes from green back to white, indicating that it is no longer a standard. To de-select all the standard points at once, click on the **Remove items** button in the toolbox.
To change the value of a standard band, click on it once in the spreadsheet. Insert the new value and select Enter on the keyboard to input the value. The value will be updated in the graph with a new value calculated for any unknown band if appropriate.

Displaying the Curve

If the box displaying the standard curve obscures a part of the image, it can be resized using Windows® operating system functions. It can be hidden from view by clicking the Graph checkbox in the Standard Curve toolbox.

Quantization Values of Unknown Bands

As the values for the standard bands have been entered, the quantization values of the unknown bands are automatically calculated. The calculated values of the unknowns are automatically updated in the spreadsheet. It is possible that the graph display may need to be refreshed after changes in standards values, adding/deleting regions, etc. To refresh the graph display simply uncheck and recheck the graph control checkbox on the Std Curve tab.

Note: The graph is based on a several different graph equations (shown in the Toolbar).

Band	Red BC Average	Red ng
1 s	10,200	100.00
2 s	4,578	40.00
3 s	6,871	65.00
4	7,020	67.55
5	1,345	-
6 s	7,348	75.00

Figure 5.32 Multiplex Band Analysis Data Box

The third column in the data box contains values for the standards (entered by the user) highlighted in green, and the values for the unknown bands (calculated based on the curve). The heading of this column reflects the units entered as in ***Specifying the Quantization Units*** above.

Standards have an “s” next to the object number, and are highlighted in green to differentiate them from unknown bands.

Values that are outside the range defined by the standards are not quantified and simply have a dash entered in the corresponding column.

Quantization Values of Unknown Bands across Color Channels

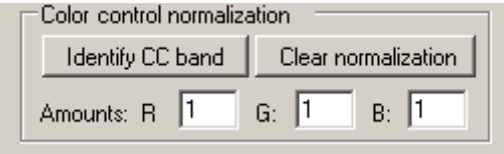
A specially constructed control sample (Color Channel Normalization Control – or CCN) is required to make quantitative comparisons between different color channels in a multicolor image. One such sample is a mixture of a single type of protein or appropriate size (similar in size to the other proteins of interest) that has three different fluorescent labels. The GE DIGE minimal dye labeling kit can be used to label aliquots of the protein with CY2, CY3 and CY5. The aliquots are then mixed together in equal amounts and loaded into a lane. When the gel is run and transferred to a membrane the resultant band generates signals in each color channel and serves as a reference.

The Color Control (CC) permits making a correspondence between a specific amount of signal from one color channel to the other color channels. Given that each channel may be acquired with different exposure times, excitation energy variations, emission filter variations the actual signals can be quite different. By normalizing the respective signals to the Color Control all the variations are removed. This permits direct comparison of image data from multiple imaging systems possible.

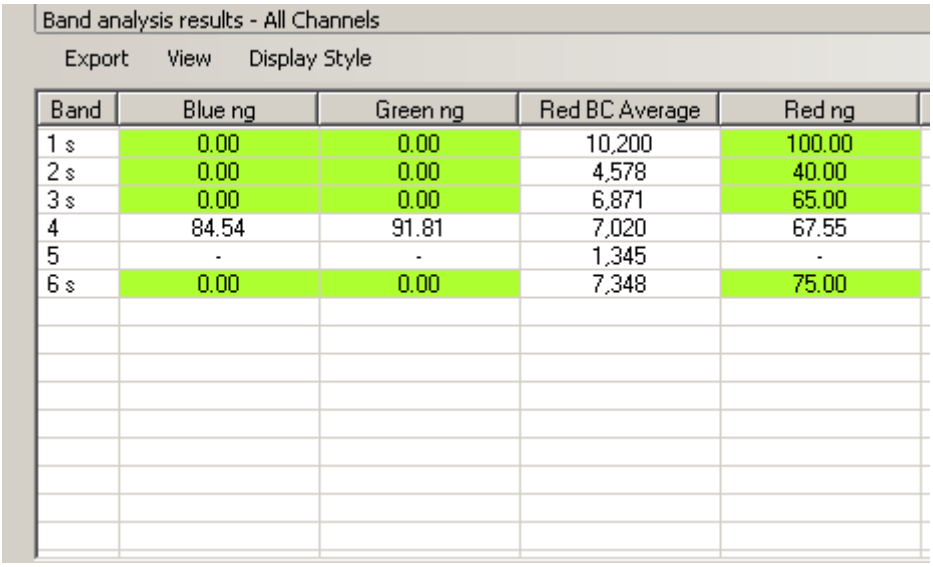
A simple example illustrates the need for a CCN: Image a blot and determine the blue/green ratio for a band. Reimage the blot with different exposure times and notice that the blue/green ratio is now different. The signal levels from the Color Control change in direct proportion to the exposure times. Using the Color Control signals to normalize the experimental band signal levels corrects for the different exposure times and resolves the discrepancy.

Identify CC Band

Select the region containing the Color Control Band and enter the relative amounts of material in each color channel.

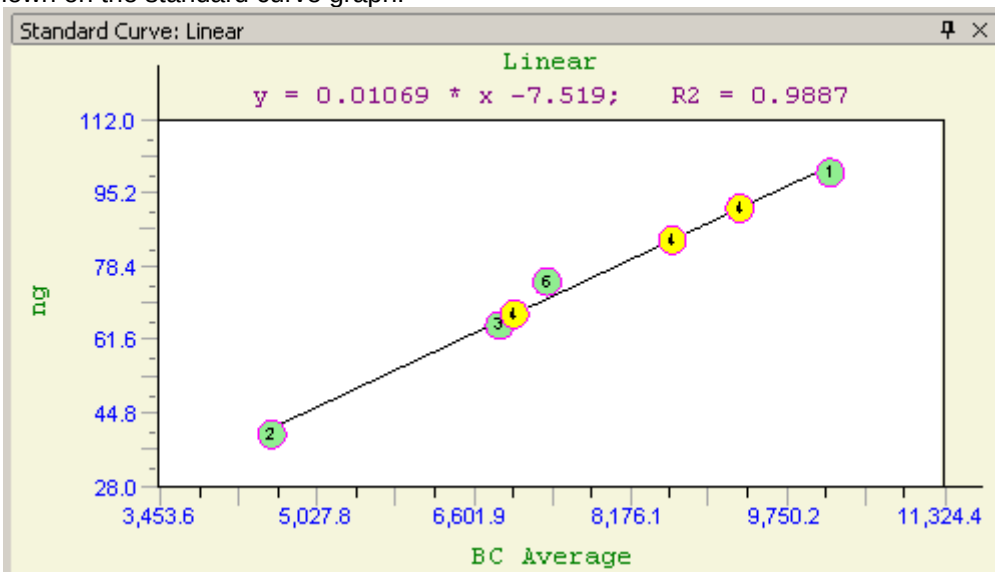


In the case of using equal molar concentrations of DIGE minimal dye labeled materials the relative amounts are 1:1:1 for CY2, CY3 and CY5. A standard curve has been defined in the red channel using a dilution sequence. Using the signals from the CC band and the known relative amounts of material in the CC band the color channel data is normalized across color channels and the unknown quantities for the green and blue channels is determined.



Band	Blue ng	Green ng	Red BC Average	Red ng
1 s	0.00	0.00	10,200	100.00
2 s	0.00	0.00	4,578	40.00
3 s	0.00	0.00	6,871	65.00
4	84.54	91.81	7,020	67.55
5	-	-	1,345	-
6 s	0.00	0.00	7,348	75.00

In the figure above band 4 now shows ng quantities for each color channel. These quantities are also shown on the standard curve graph.



See Appendix X for a detailed analysis shown use of CCN to determine the amount of phosphorylated versus non-phosphorylated protein isoforms.

Lane Profile (Lane Densitometry)

The Lane Profile button in ToolBox, Analysis Tools accesses a set of densitometry tools with which bands on a gel can be scanned and analyzed in a lane format. There are two different ways in which this can be done, **Auto Lane** and **Auto Grid**. Auto Grid allows the user to manually define the lane number, lane shape, and scan width of the Grid. Auto Lane is a completely automated feature which will automatically define lane number, and band finding parameters for the user. Both detection methods provide similar data. Image particulars and user preference will determine which method works best.

Auto Grid

When the Lane Profile tab is selected the Auto Grid template appears on the image and the following functions are displayed:

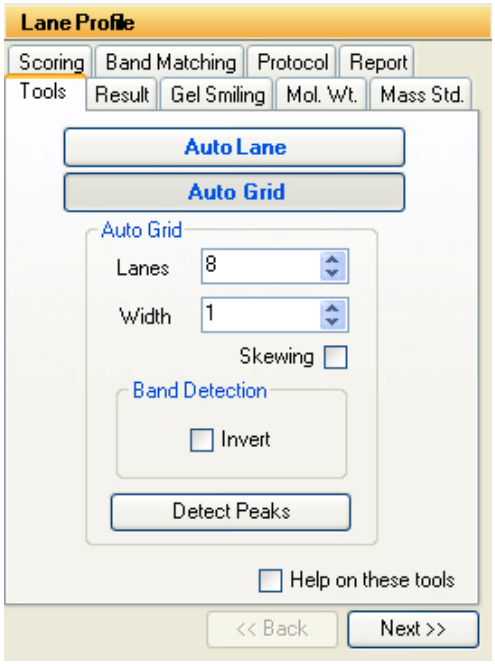


Figure 5.33 Lane Profile tools

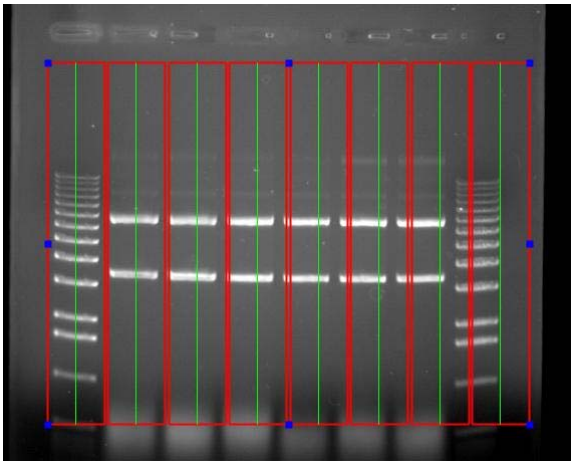


Figure 5.34 Lane Profile Template

Setting up the Lane Template

When the Lane Profile button is clicked, a lane template appears on the image. The red lines indicate the borders of adjacent lanes. The green lines define the Scan Width (described below).

Under the heading **Grid Controls**, the number of lanes can be specified. The number under the title Lanes indicates the number of lanes and can be changed by clicking the right (increase) or left (decrease) arrows. As the lane number is adjusted, the template displayed over the image is updated to reflect the changes.

Once the number of lanes has been set, the template should be adjusted so it coincides with the lanes on the image.

If the lanes on the gel are horizontally oriented (sideways) the template can be rotated. To change the orientation of the template to coincide with the image, click the ROTATE checkbox. The template will rotate 90° counterclockwise. Clicking in the check box again ("X" disappears) rotates the template 90° clockwise, restoring it to its original orientation.

After specifying the correct number of lanes and setting the orientation, use the mouse to drag the outside borders of the template so they frame the lanes to be scanned. Clicking on or within any border repositions the template. Clicking on the blue “handles” surrounding the template resizes it.

If the lanes of the gel are not perfectly straight, click the SKEWING checkbox. The template will change to show four “handles” -- one at each corner of the template. Position each corner of the template until it is positioned appropriately.

Adjust the template until the border of the template frames the lanes to be scanned and the red lines lie between each of the lanes.

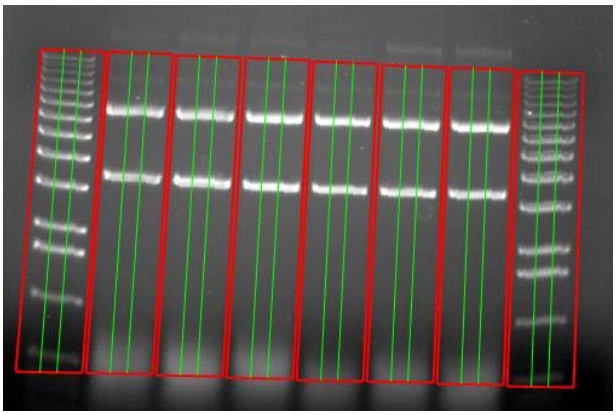


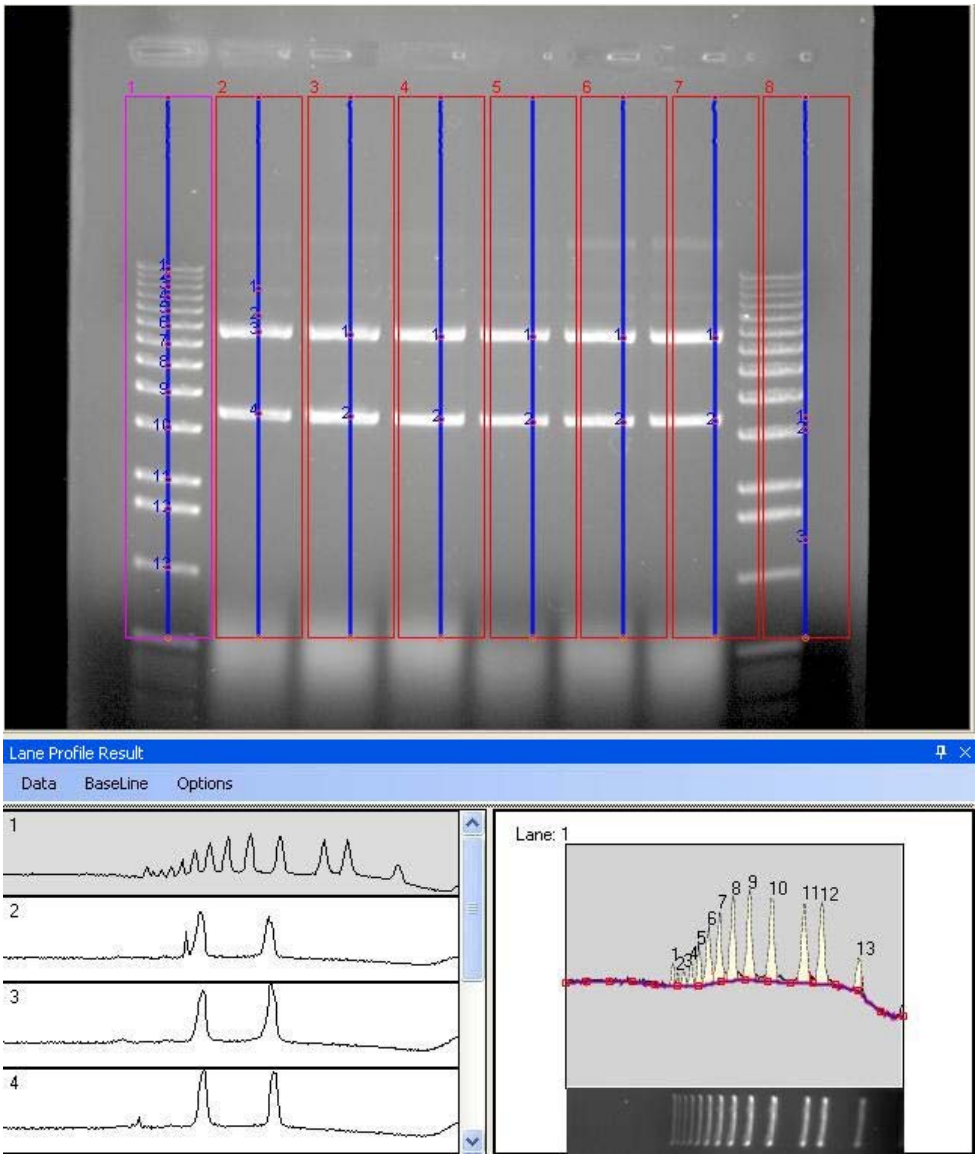
Figure 5.35 Skewed Lane Profile Template Properly Aligned on a Gel

The Invert check box should be selected if the image being analyzed is a dark sample on a white background. These are usually colorimetric gels such as Coomassie Blue. Reversing the image on the Contrast Adjustment window would NOT require the user to select invert.

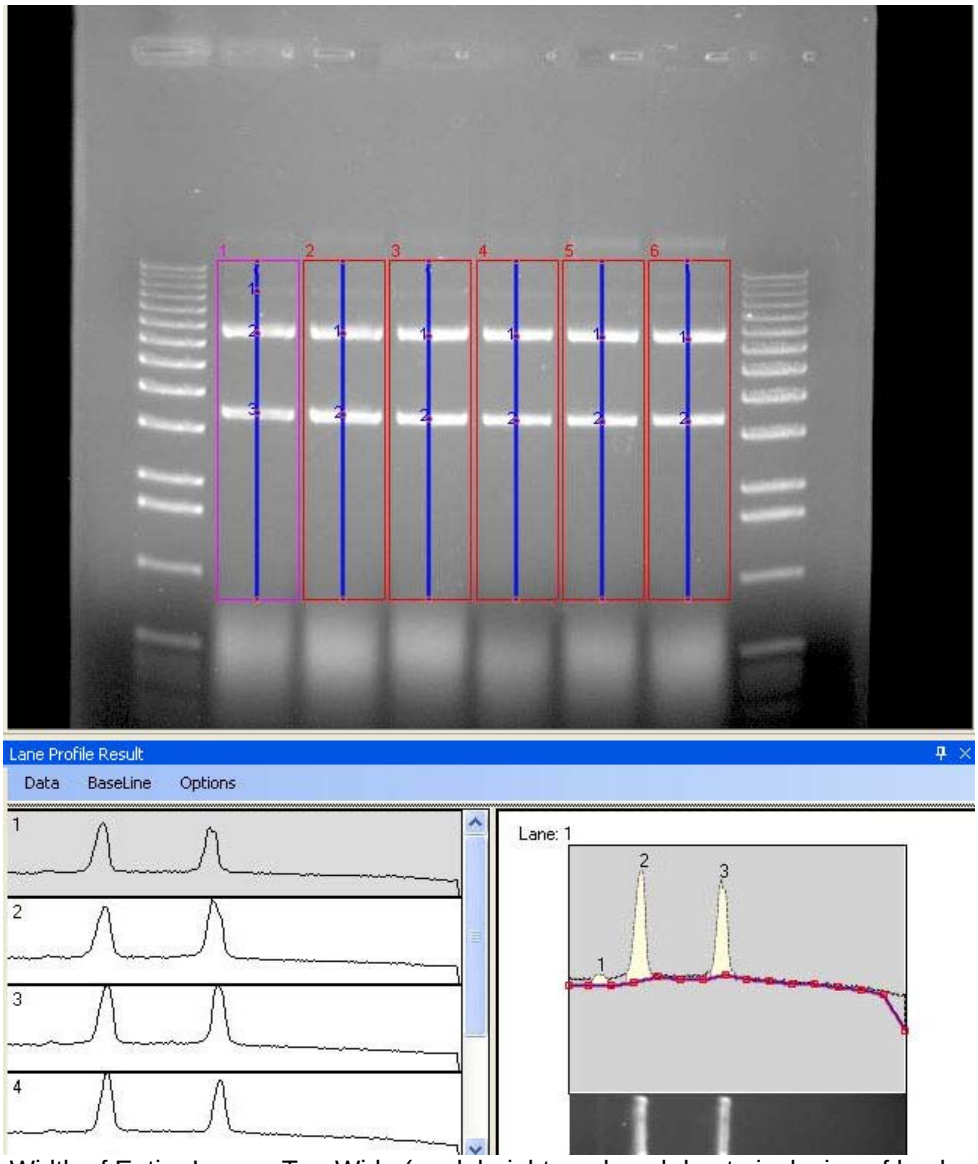
Specifying the Scan Width

The Scan Width (the green line within each lane) is the area from which the pixel density is measured. The control labeled WIDTH sets the Scan Width. The number in the center of this control indicates the current Scan Width setting. It can be adjusted by clicking on the right (increase) or left (decrease) arrows. As the setting is changed, the template displayed on the image is updated to reflect the changes.

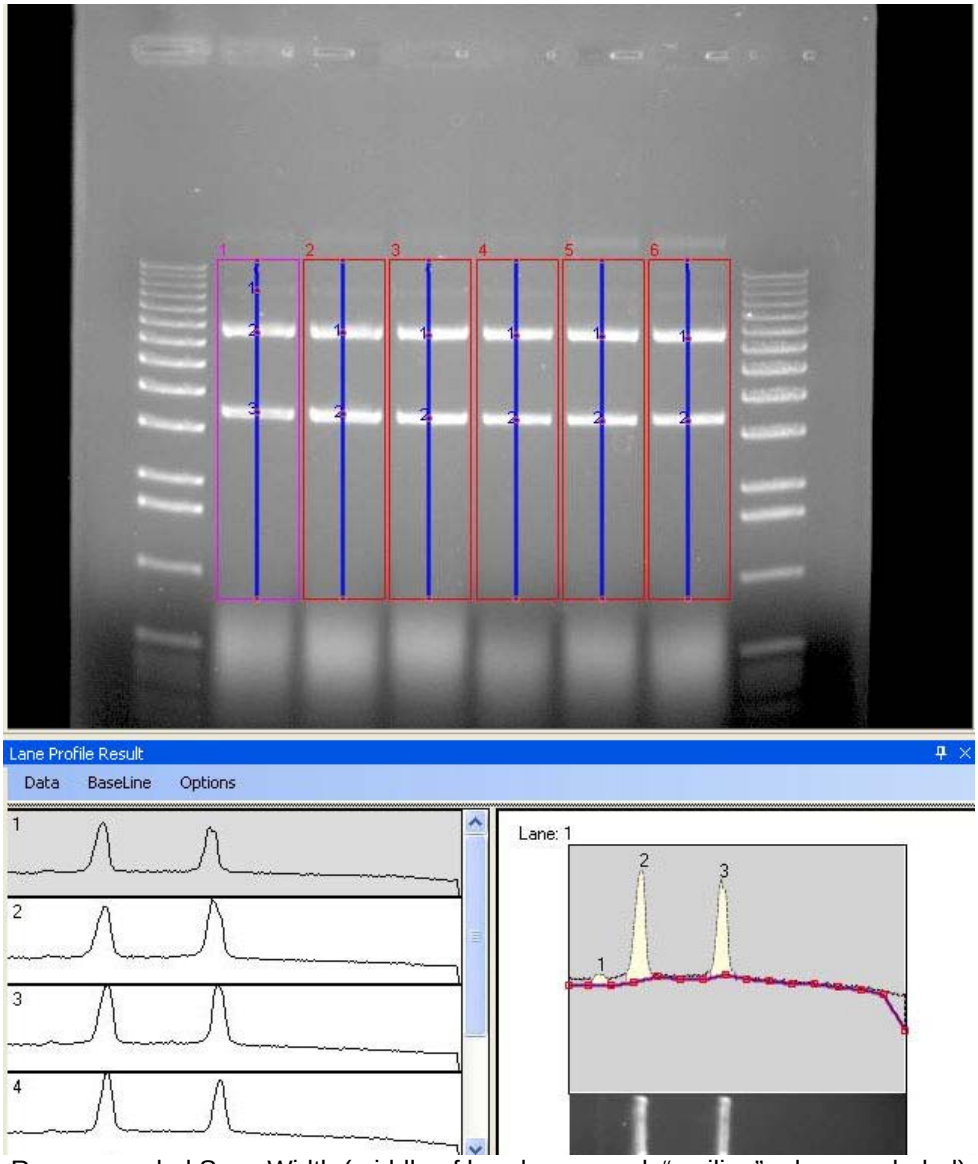
The density values for Lane Profile graphs are determined by the *average* pixel density between the green lines on the template. A scan width that is too narrow may not include enough information and can result in a noisy graph. By contrast, a wide scan width can incorporate background pixels that will reduce the pixel average and dilute the actual data. Therefore, to generate the most meaningful quantitative data, set the width to include as much of the bands as possible without including regions of background. The scan width should not exceed the band edges and cannot exceed the red lane limit.



Scan Width of 3 = Too Narrow (data noisy and graphs jagged)



Scan Width of Entire Lane = Too Wide (peak heights reduced due to inclusion of background pixels)



Recommended Scan Width (middle of bands scanned; “smiling” edges excluded)

Scanning the Image

When the AUTOGRID button is clicked, the densities of each lane are measured, and information is displayed in several quadrants of the screen:

- The graphs for each lane scanned are displayed in the lower left.
- The active graph is displayed in the upper quadrant of the screen.
- Peak information for the active graph is displayed in a data table in the lower right.
- The Image is displayed behind the above windows and can be viewed by reducing the individual display windows.

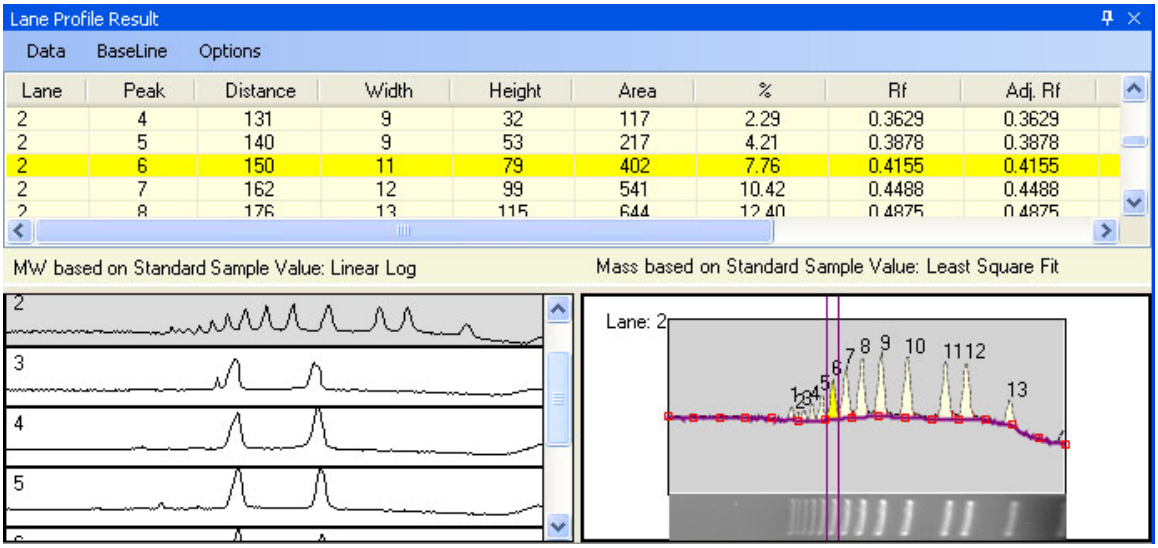


Figure 5.36 Image Area of Sample Scan

Graph Display

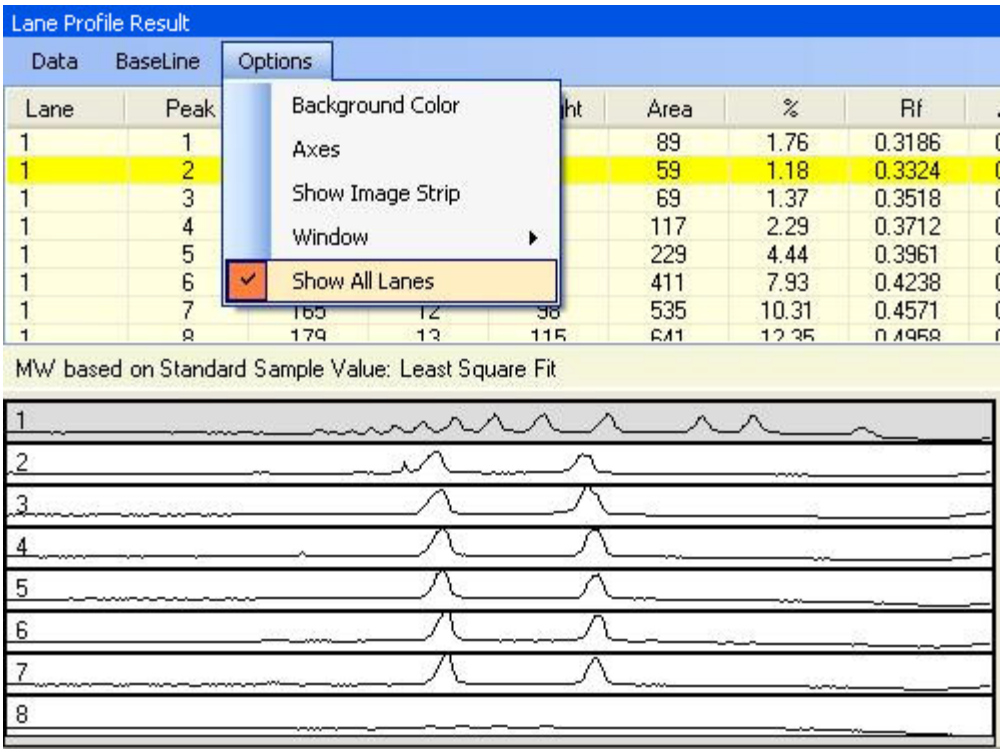
Graphs representing all the lanes on the template are shown in the lower left-hand quadrant.

When AUTOGRID is first selected, the active graph is the one corresponding to Lane 1. To select a different lane, simply click on its graph, and it will fill the Active Graph quadrant.

AlphaView uses the following color-coding:

- the active graph is shown in white
- graphs that have been viewed already are shown in yellow
- graphs that have not yet been viewed are shown in gray

When the template contains many lanes, there may be too many graphs to see reasonable detail. It is possible to view only four graphs at a time by deselecting Show All Lanes. This function is found under the function button of the window.



Deselecting Show All Lanes

The Active Graph

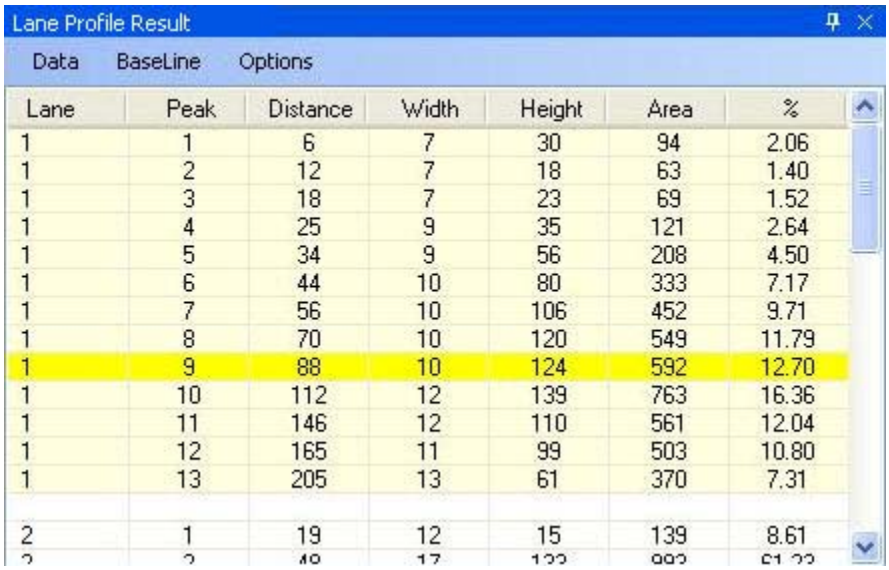
The active graph is shown in the upper quadrant. The x-axis represents the distance (in pixels) from the top of the template. The y-axis represents the average pixel intensity across the width of the scan.

Note: Lane scans are shown without axes in order to show as much detail as possible in a small window. To show the graph's axes, choose Axes from the Options pull down menu. To change the background color of the graph, choose Background Color from the Options pull down menu.

AlphaView automatically detects the peaks and integrates the area under each peak. Adjustments that can be made manually are described below.

The Data Window

Once a peak is defined, its integration data and associated information are displayed in a table located in the lower right quadrant of the screen. The data table is updated any time a peak is deleted or added, peak boundaries are redefined, or the background value is re-set.



Lane	Peak	Distance	Width	Height	Area	%
1	1	6	7	30	94	2.06
1	2	12	7	18	63	1.40
1	3	18	7	23	69	1.52
1	4	25	9	35	121	2.64
1	5	34	9	56	208	4.50
1	6	44	10	80	333	7.17
1	7	56	10	106	452	9.71
1	8	70	10	120	549	11.79
1	9	88	10	124	592	12.70
1	10	112	12	139	763	16.36
1	11	146	12	110	561	12.04
1	12	165	11	99	503	10.80
1	13	205	13	61	370	7.31
2	1	19	12	15	139	8.61

Figure 5.37 Example of a Quantitation Data Table

PEAK is the number assigned to each peak on the graph beginning on the left and moving right.

DIST is the distance (in pixels) that each peak starts from the beginning of the line scan.

WIDTH and **HEIGHT** refer to the size of each peak.

AREA is the integrated area under each peak and represents band intensity.**%** is the percentage each peak contributes to the total density. (The sum of this column will be 100% for each lane).

Adjusting Peak Detection Parameters

Minimum Area, Height and Width

Once the graph to be analyzed has been selected and is displayed in the upper right of the screen, the tools in the lower left of the screen change to allow adjustment of the automatic peak finder parameters.

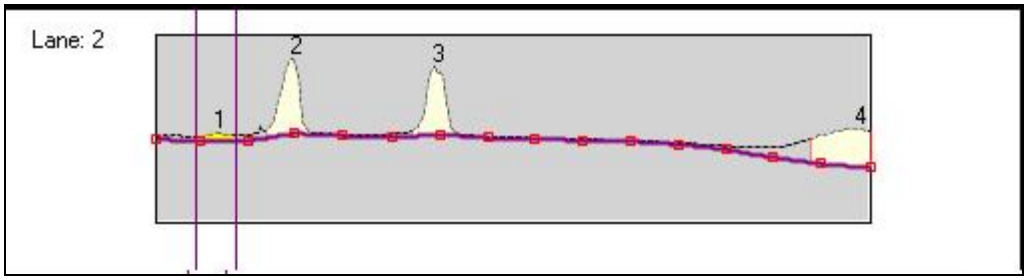
Figure 5.38 Tools For Adjusting Automatic Peak Finding Parameters

There are three attributes that determine whether or not the automatic peak finder recognizes a region of the graph as a peak: minimum area, minimum height and minimum width. Click on the

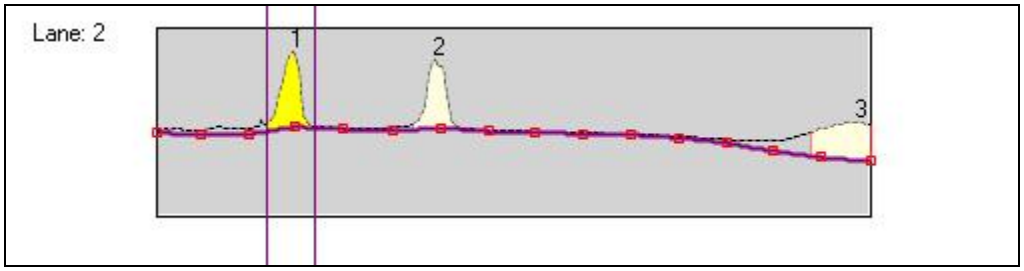
arrow(s) associated with the MIN. AREA, MIN. HEIGHT and MIN. WIDTH headings to adjust these settings.

As these parameters are adjusted, the automatic peak finder rescans the graph and defines the peaks based on the new settings. A peak is recognized only if it meets these minimum criteria.

Note: Using the MIN. WIDTH control is the quickest way to regulate the number of peaks detected by the automatic peak finder.



Minimum Width is 10



Minimum Width is 17

Editing Peaks

Editing Peak Boundaries

The boundaries of existing peaks can be readjusted to make the peaks wider or narrower. To adjust either peak boundary, click on the peak to select it. Next, point the cursor at the vertical boundary line, hold down the left mouse button and drag the line to the desired location. When it is in place, release the mouse button.

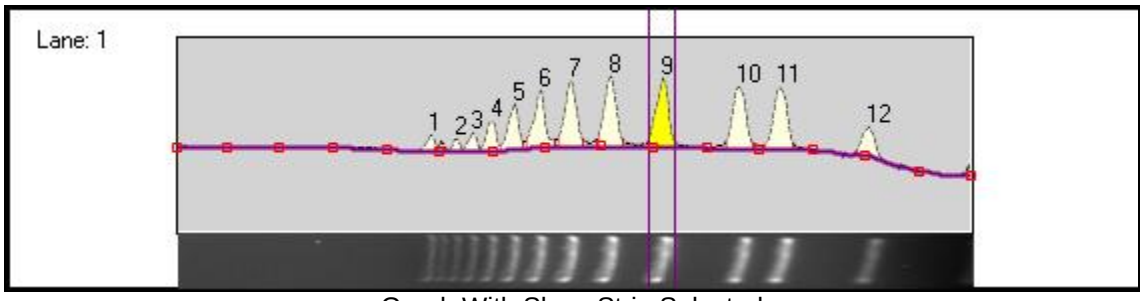
The data table is automatically updated to reflect any adjustments. Both the peak and the data in the data table will be blue to indicate that this is a user-defined (vs. automatically detected) peak.

Manually Adding Peaks
Additional peaks can be defined manually using the Add Peak function (in the Integration menu). When this function is selected, a vertical line appears. Position the vertical line so it corresponds to the left edge of the peak, click the left mouse button to define the left boundary of the peak, then move the cursor to the right edge of the peak and click again to define the right boundary. The peak area between the 2 limits now appears shaded, and the data table is automatically updated. Both the peak and the data in the data table will be blue to indicate that this is a user-defined (vs. automatically detected) peak. To define a second peak, click the right mouse button to reactivate the cursor.

Deleting Peaks
A peak can be deleted by clicking on the peak and selecting Remove Peak from the Integration menu. The selected peak's integration disappears, and the integrated peaks to the right are renumbered accordingly.

A peak can also be deleted by clicking on its entry in the data table window and hitting the <Delete> key on the keyboard. To prevent accidental deletions, a dialog box will open, asking for confirmation.

Visualizing Peak Placements
To determine exactly where a peak is located relative to the original image, it is often helpful to see the graph and the gel image in the same orientation. The Show Strip function (found in the Options menu) displays the area of the gel that has been defined as a lane across the length of the x-axis.



Graph With Show Strip Selected

Note: If Show Strip fails to show a portion of the image, check that the appropriate driver for the VGA card is selected. (See Appendix X or Section 1.3 for more information.)

Resetting Peak Edits
The Auto Peak function in the Integration menu recalls the automatic peak finding algorithm used when the graph was first opened. Note: If the Auto Peak Detection Parameters (height, width and area) have been changed, these will be the variables used with the algorithm, and the peaks may differ.

Clearing All Peaks
To delete all the defined peaks on a graph at once, click on the Clear All Peaks function in the Integration menu.

Adjusting the Baseline

As a default, the baseline value is set to Auto Base. As the baseline is adjusted, the values for all peaks are updated to reflect the new baseline.

All of the following functions are found in the Background Subtraction menu:

Auto Base
This method breaks the red line defining the left boundary of the lane into sixteen regions, and outputs sixteen points which reflect the average backgrounds across those sixteen regions. The user can manipulate the 16 points by clicking and dragging. The user may also designate more points than the 16 and manipulate those in the same fashion.

Intra-Lane
This method uses the lower of the two values at the edges of the lane along its travel as the background. If the section of the image is used is wider than the bands, then this is a very effective way of removing co-migrating material from your measurements. This method should only be used if the bands are completely enclosed. This is because when the edges of your lane area intersect any of your bands, band material will be considered as background, severely affecting your results.

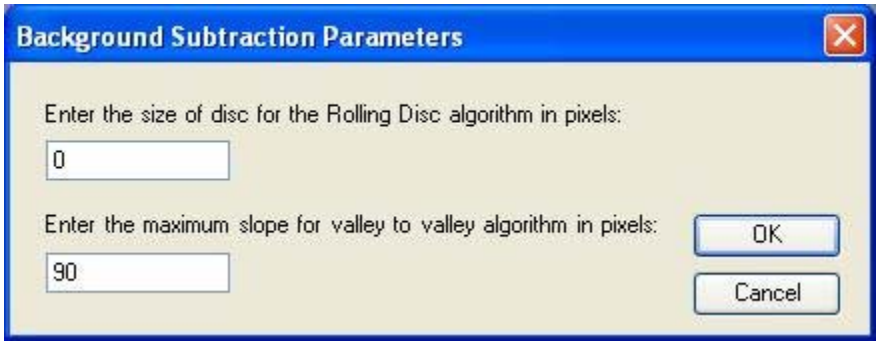
Rubber Band
This method can be thought of as stretching a rubber band underneath the lane profile. This option does not work well if the values at the ends of the intensity profile are lower than the rest of the intensity pixels. We do not recommend this method for images with poorly separated bands.

Minimum Profile
This method takes the lowest value on the profile of each lane as the background for that lane. We do not recommend this method for lanes where the beginning or end of the lane is the lowest point as this is dependent on where the lane boundary was placed when it was defined. This can be hard to repeat between analysis of the same gel.

Valley to Valley
This method requires that you have performed band detection first. The background is taken as the line between the edges of the bands in the lane. You enter a value (see below in setting parameters) of maximum slope in the accompanying entry box to avoid situations where the edge between overlapping bands, which is not at the background intensity, causes the background to climb too high.

Rolling Disc
This method requires you to enter a parameter for the size of the disc (see below in setting parameters) in the entry box. This option calculates the background as if a disc, with the radius you have entered, were rolling underneath the lane profile. The larger the radius of the disc, the less the background rises with the profile.

Set Parameters:



Set parameters dialog box

Above dialog box is used to change values for Rolling Disc and Valley to Valley background methods.

Base Lock

The Base Lock function will designate the active graph baseline for all of the lanes in the data table. To de-select this option click on the Base Lock a second time. The check mark will disappear.

Horizontal Base

Activating the Horizontal Base function will move the baseline to the 0 position (no background). The baseline can be moved vertically by clicking the baseline and dragging it upwards.

Reset Base

Activating the Reset Base function will move the baseline to the 0 position (no background). Unlike Horizontal Base the baseline cannot be moved.

Base Subtract

This selection will subtract the current baseline from the intensity value line. Un-check Base Subtract to deactivate this feature.

Interpreting Lane Profile Data

AlphaView provides a variety of tools to help interpret Lane Profile scan data. These tools are found in the toolbox at the bottom of the screen, and in the Data pull down menu in each graph window.

Figure 5.39 Lane Profile Data Interpretation Tools

V. Line

Typically, the peaks on a graph correspond to the bands in a lane. The V. Line tool can help determine if the peaks on two different graphs represent bands at the same molecular weight position (i.e. the same distance from the top of the gel).

To compare the positions of peaks on different graphs, click on the V. Line checkbox. A vertical line which can be moved across the graphs will appear. Use this line to determine if two peaks are located in the same position

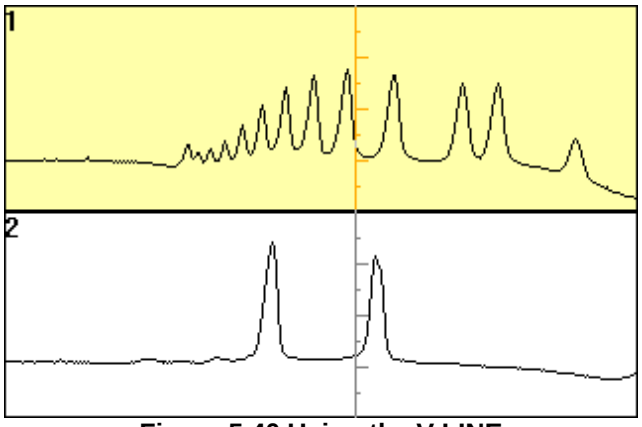


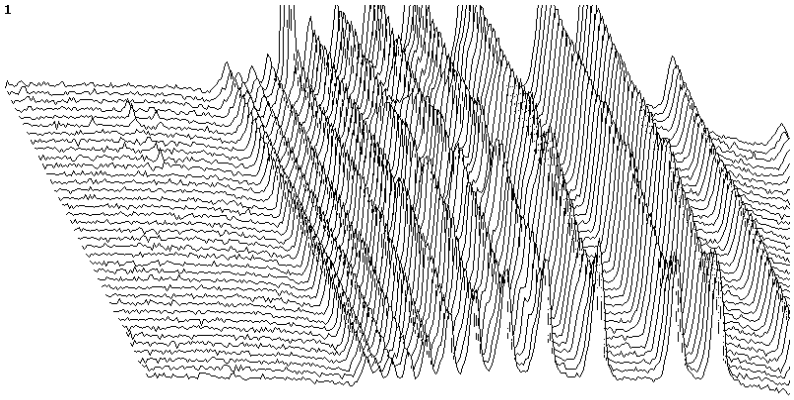
Figure 5.40 Using the V.LINE

The line also contains hash marks, which represent pixel intensities. Use this information for a quick comparison of peak heights. Note: The hash marks are most useful when only four graphs are shown so that enough detail is displayed. To accomplish this, click on the selection button in the upper left corner of the window and deselect Show All Lanes.

Alternatively, the intensity value at the point where the V.Line crosses the graph can be displayed. Hold the <shift> key and click the right mouse button to toggle between intensity values and hash marks.

3-D View

The 3-D View feature changes the appearance of the graphs to a 3-dimensional display. This can be useful in understanding how data was obtained, and in visualizing any anomalies that occur on the gel.



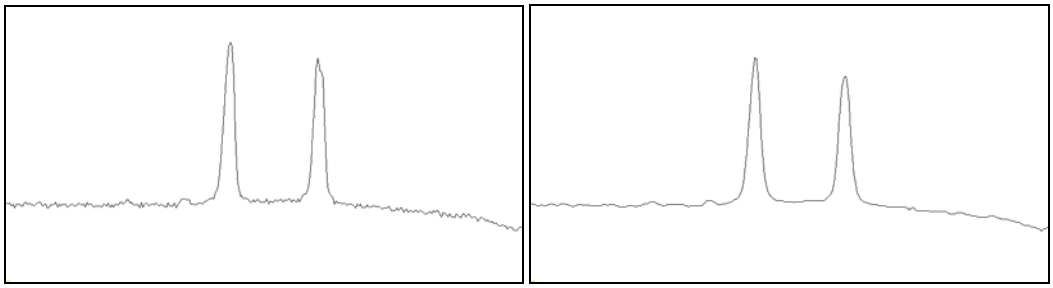
3D Scan

The depth of the display is determined by the Scan Width (i.e. the above scan has a width of 35 and is displayed in 3-D mode as 35 graphs). This function acts like a toggle: click in the box next to 3-D View to activate the function; click in the box again to deactivate it and return the graphs to their original appearance.

Note: If a lane has been scanned with a large width, it may take a long time to display the graph in 3D mode. If this is the case, select 3D View: Show Hidden Lines from the utility menu in the graph window. This will result in a graph that is not as "clean," but which displays on the screen very quickly.

Smooth Data

The Smooth Data function (found in the Data menu) minimizes single pixel background noise in a lane.



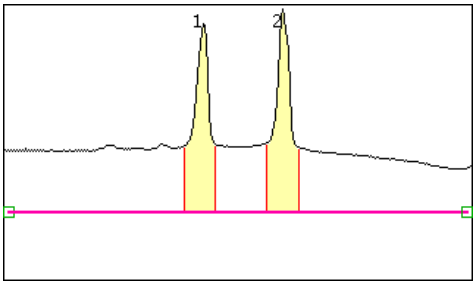
original graph smoothed graph

When this function is selected, the menu option changes to "Unsmooth Data." Selecting this undoes the smoothing operation.

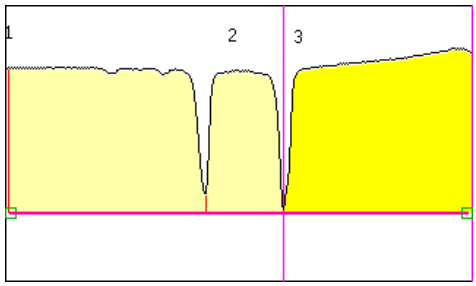
The Invert Button
The INVERT function reverses the gray scale assignments, so that zero corresponds to white and 4,096 corresponds to black. This function can be selected by placing an "X" in the INVERT checkbox, or by selecting Invert Data from a graph's Data pull down menu.

If the image has dark bands and light background, then INVERT should be selected. If the image has light bands and dark background, the INVERT option should not be activated.

On the graph, the regions that were peaks are now closer to the baseline and those that were near the baseline are now peaks.



original graph



inverted graph

Since the baseline is determined when the image is initially scanned, it may be necessary to use the Reset Baseline function to set an accurate baseline for newly-inverted data.

When the Invert Data function is activated from the pull down menu, the menu option changes to "Uninvert Data." Selecting this undoes the inverting operation.

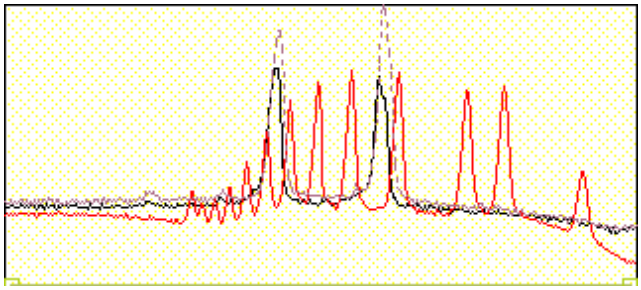
Unlike the REVERSE button described in Chapter 2, this function does not alter the appearance of the image, but does change the data.

Overlaying Graphs
When comparing the bands in one lane with those in another it is often convenient to display the graphs so that one is superimposed over another. After clicking on a graph so that it is enlarged in the image area, select the Overlay Control function (from the Data menu) to display the Lane Overlay Control dialog box. In this dialog box, specify the lane(s) whose graph(s) should be superimposed and the color and line pattern in which they should be displayed.

- The first column in the dialog box contains the numbers of the graphs which can be superimposed.
- The second column reports whether or not the graph of a particular lane is currently superimposed. These ON/OFF settings toggle back and forth by clicking on them.
- The colored bar in the third column indicates the color in which a graph will be displayed when it is superimposed. To select a color option, click on the color bar until the desired color is shown.
- The last column in the dialog box specifies the pattern of the line when the graph is superimposed. To select a pattern, click on the current pattern until the desired pattern is shown.

It is especially important to choose different patterns if the graphs will be printed, as different colors cannot be distinguished using a gray scale printer.

When the selections have been made, click on the DONE button to dismiss the dialog box and superimpose the selected graph(s). (If no graphs have been selected, the dialog box is simply dismissed.)



Three Graphs Overlaid

To turn all graph superimposing off, click on the ALLOFF button at the bottom of the dialog box.

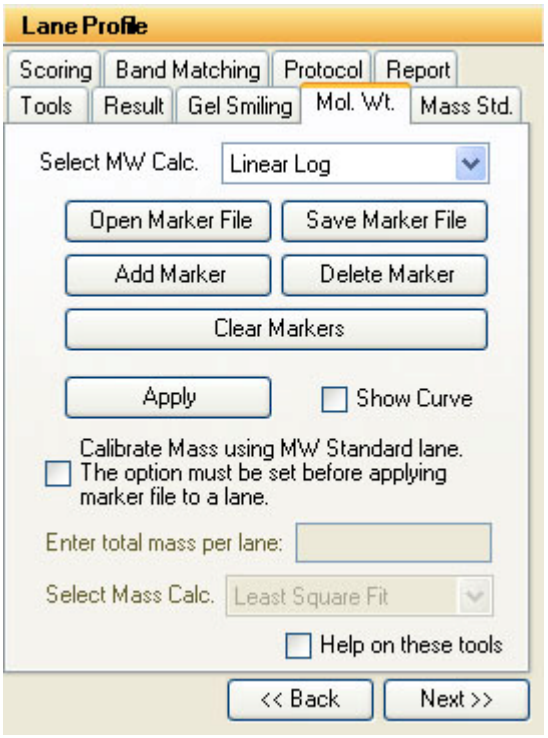
Molecular Weight, Mass and Band Scoring integrated into Lane Profile

A new menu item has been added to Lane Profile. Under this menu the user now has the option of calculating Molecular Weight and/or Mass. The user can also access the Band Scoring feature from this menu. This unifies four important analysis tools into one area of the software and reports Mass, Gel Smiling, and MW on one report.

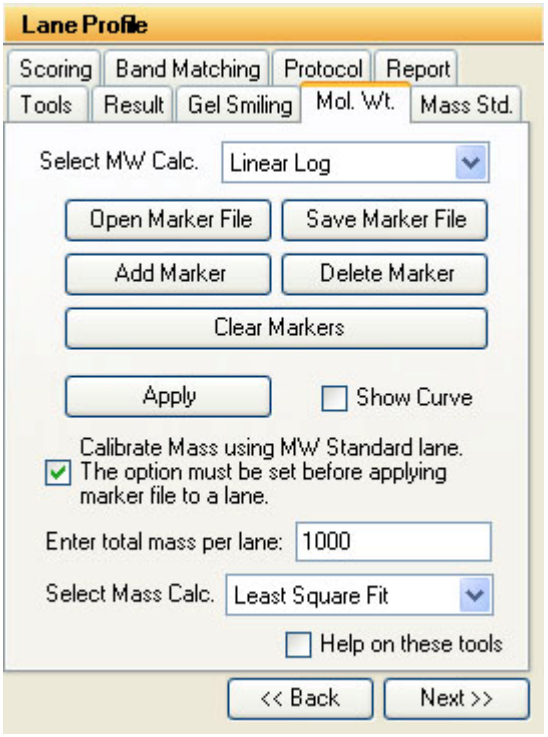
Figure 5.41 Molecular Weight, Mass and Band Scoring integrated into Lane Profile

Molecular Weight and Mass integration

AlphaView supports Mass calculations based on the users MW standard. Note the new Checkbox in the Molecular Weight Standard dialog window labeled “Calibrate Mass using MW std lane”.



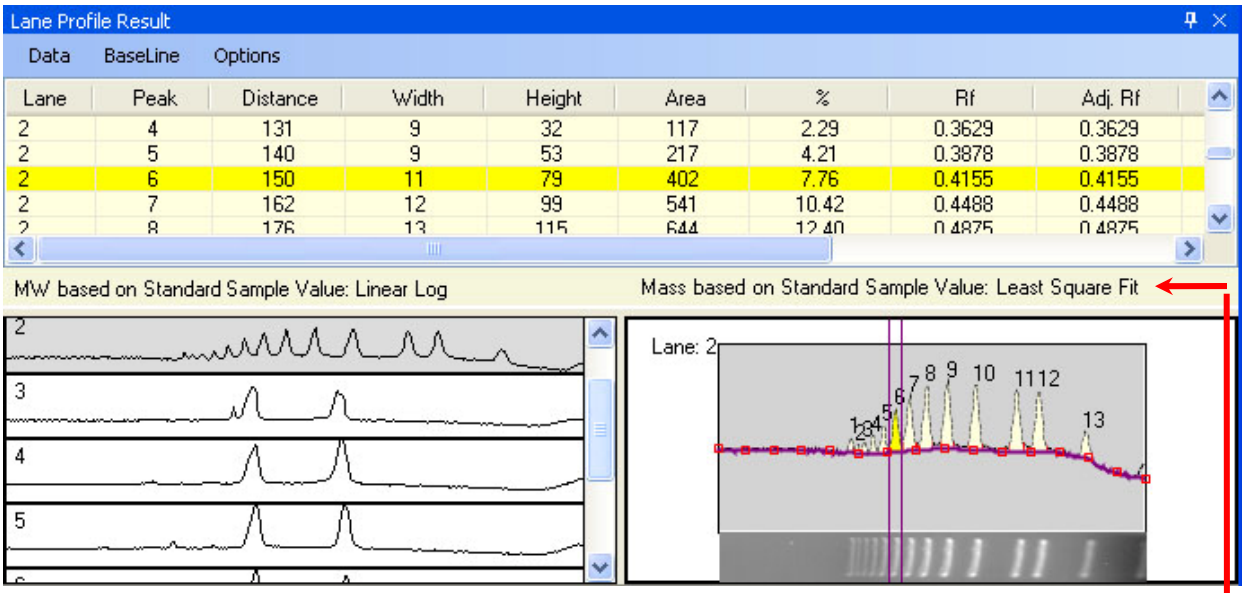
Checking the checkbox activates the “Enter total mass per lane” field and reveals the choices for calculating the Mass values.



Procedure:
If you would like to calculate Mass based on your MW Standard Lane follow these steps.

1) Check the “Calibrate Mass using MW std lane” checkbox.
2) Enter the total mass that was loaded into the MW standard lane into the ‘total mass per lane’ field.
3) Select the Calculation method to be used for Mass calculation.
4) Click the Open Marker or Add Marker buttons and apply MW standard markers in the normal way.
5) Click OK or Apply.

The Mass for all bands is now calculated based on the total mass entered for the MW standard lane and reported in one report.



Note Legend: Describes which lanes/bands make up the MW and Mass standard curve values. Also notes which regression method has been used to calculate the MW and Mass curves.

mws: Molecular Weight Marker
ms: Mass Standard Marker

Saving and Loading Mass Standards

Mass standard files can be loaded onto any image and saved for future reference. To load a marker, select Mass Standard from Lane Profile. A Mass Standard tab will show on the screen:

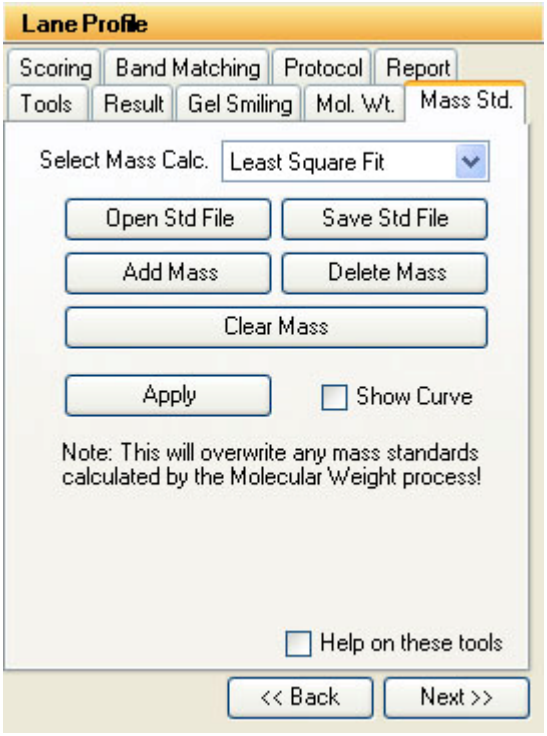
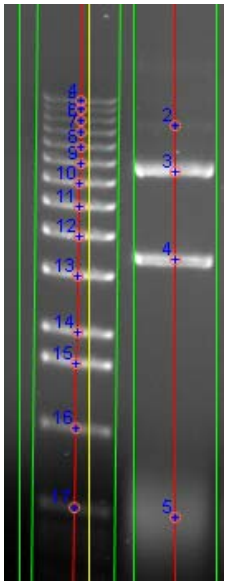


Figure 5.42 Saving and Loading Mass Standards in Lane Profile

Select Add Mass Std to add the first marker. Pull the cursor onto the image to select the band of interest and click once on the left mouse button to add the marker. Type the marker value into the dialog box. Continue this process until all of the standards have been loaded.

After the mass standards have all been entered, select Save Std File. Then move the cursor to the lane that contains the markers of interest. A yellow line will appear in autolane. To select the lane of interest click once on the left mouse button and a new dialog box will appear allowing for the name of the file to be saved in the directory of interest. Select apply to calculate the unknown values of the markers in the rest of the lanes.



Mass Standard files can be loaded by selecting Add Mass Std in either autolane or autogrid. Browse through the directories to find the marker file of interest, then select the file name and click on open. The user will have the option to add the markers manually or to auto-load the markers. Markers can be auto-loaded by selecting yes in the dialog box and then pointing to the appropriate lane on the gel in autolane, or the appropriate lane profile in autogrid. To add the markers one at a time, select no in the dialog box and then point to the individual bands and click once on the left mouse button on the band of interest. Continue to do this until all of the markers have been loaded. Select apply to calculate the unknown mass values in the rest of the image.

Band Scoring

Lane #'s to score: Choose the lanes to score by highlighting the lanes to be included.
Score based on: Select the parameter to base the scoring on. The choices are Area, Mass, and Height. The Area and Height are calculated automatically in Lane Profile. In order to use the Mass as the scoring parameter the user must calculate the Mass of every band by applying a Mass Standard. This can be done separately or in conjunction with MW calculations. See the section above on Molecular Weight and Mass integration.
Select Scoring Method: Select the scoring method of interest. The choices are: Present/Absent; High/Medium/Low/Absent; % of control; and Quantity.

- Present/Absent: this method is used as a qualitative measurement for review. The researcher is interested in whether or not a lane has a specific band of interest based on some criteria (area, mass, or height).
- Additional Criteria: When choosing Present/Absent as the scoring method the user must enter a value for determining the presence or absence. The value is a percent. When the value of an unknown band falls below that percentage value it is classified as Absent, when it exceeds the value it is classified as Present.

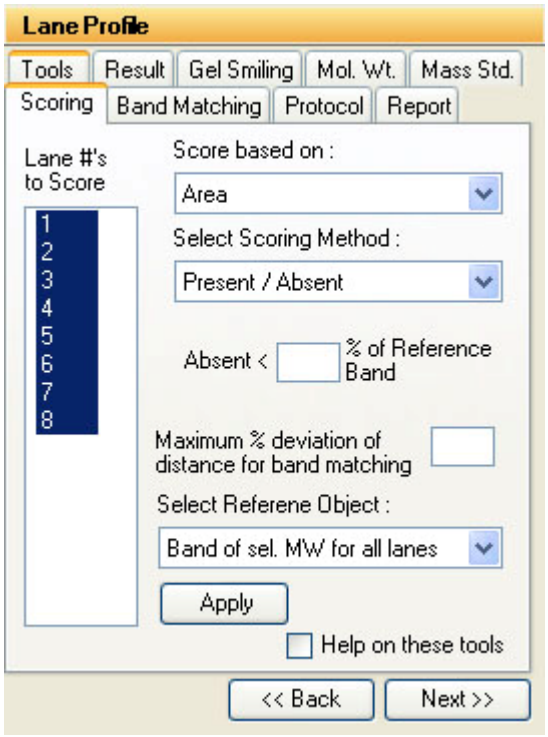
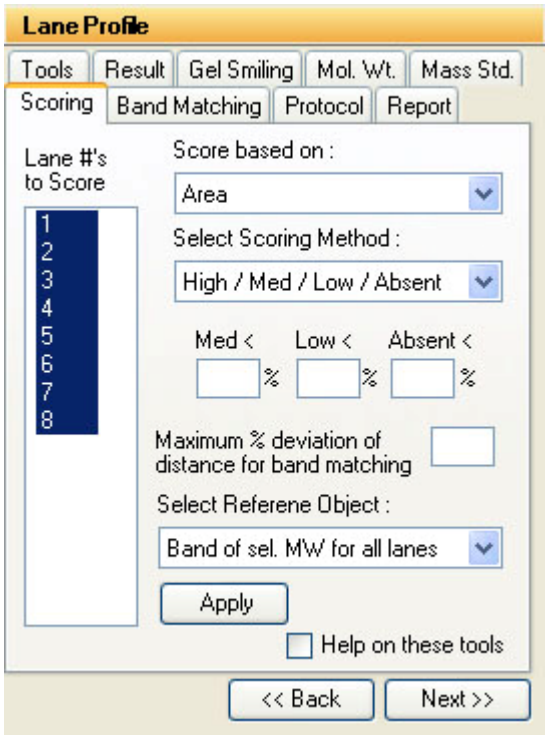


Figure 5.43 Band Scoring

- High/Medium/Low/Absent: This method is used to classify bands into groups based on Area, Mass, or Height. The researcher will determine break points for each classification (high, medium, low, and absent). The software will place each band into one of these classifications depending on the user entered criteria.
- Additional Criteria: When choosing the High/Medium/Low/Absent method the user must enter in values to use as break points for each of these classifications. The values entered are percentages of the reference band based on the parameter chosen in the "score based on" field.



Auto Lane

If Auto Lane is selected from the main Lane Profile tab the following interface will appear:

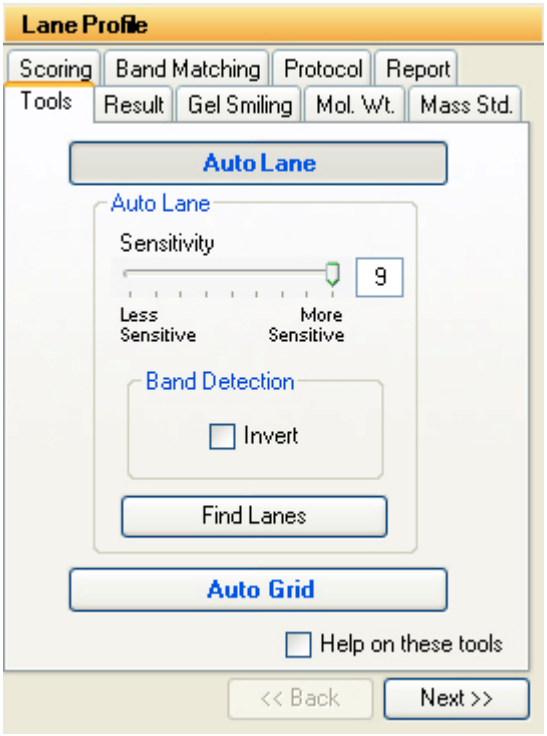


Figure 5.44 Auto Lane

Invert

Auto Lane will automatically detect whether the image about to be analyzed represents white bands on a dark background (e.g. fluorescence) or dark bands on a white background (e.g. Coomassie Blue gel, x ray film). The user should always check to see that the software has correctly characterized the image as Black or White Band. The user can override the automatic selection.

Vertical Lanes or Horizontal Lanes

If the gel image has vertical lanes the Vertical Lanes option should be checked. If the image's lanes run horizontally then the Horizontal Lanes option should be chosen. The software will not automatically detect the lane orientation so the user should aware of this parameter.

Sensitivity Adjustment Bar

This feature changes the number of bands the software will find using a sensitivity scale of 0-9. The lower the number (slider bar left) the less bands the software will find, and the greater the number (slider bar right) the more bands the software will find. Upon changing the sensitivity, Find Bands must be selected for the software to "re-find" the bands.

Area of Interest Drawing

It is recommended that the user draw an area of interest on the image in order for faster and more accurate detection of lanes and bands. An area of interest is drawn using the left mouse button to click and drag (draw) a box shaped region around the sample area. Image artifacts (well position marks etc) should be left out of the area of interest as the software may record these as bands. After an area of interest is drawn the Find Lanes button should be selected. The software will automatically find lanes and bands within each lane. An area of interest need not be drawn for the automatic detection Find Lanes to work. The analyzed image and overlaid data table will now appear.

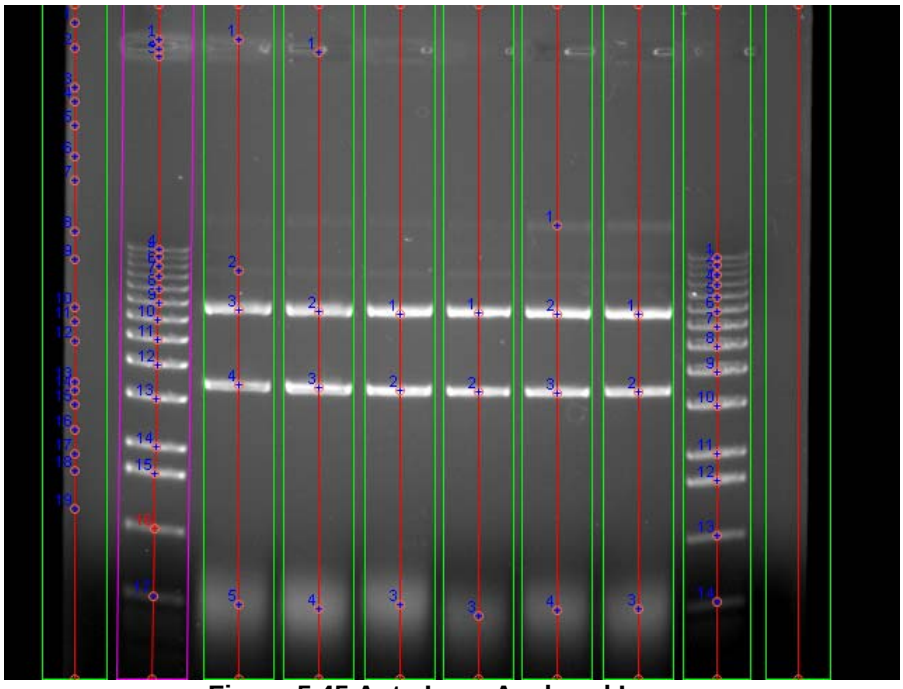


Figure 5.45 Auto Lane Analyzed Image

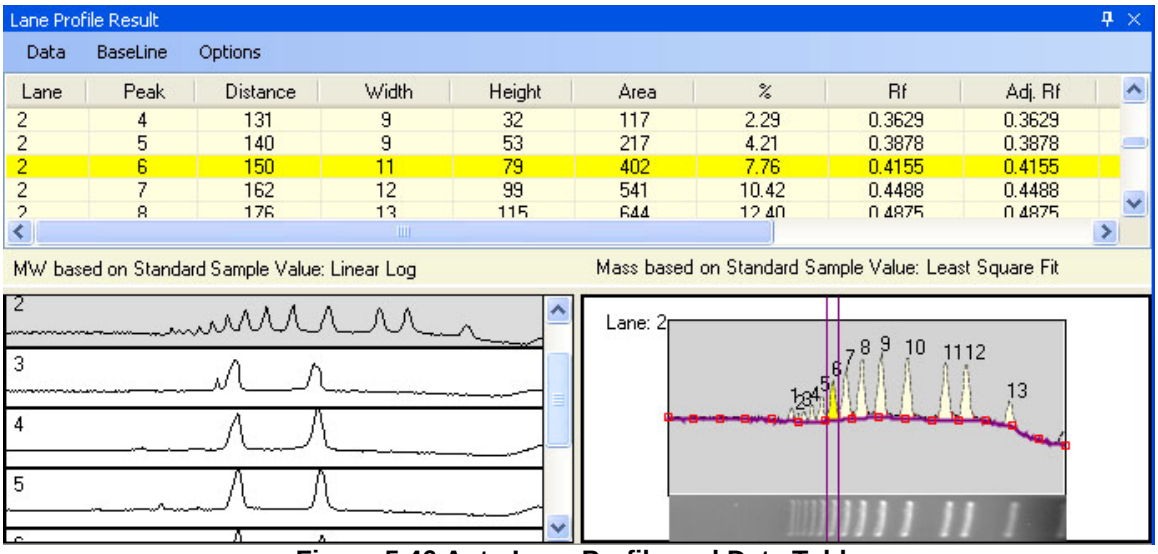


Figure 5.46 Auto Lane Profile and Data Table

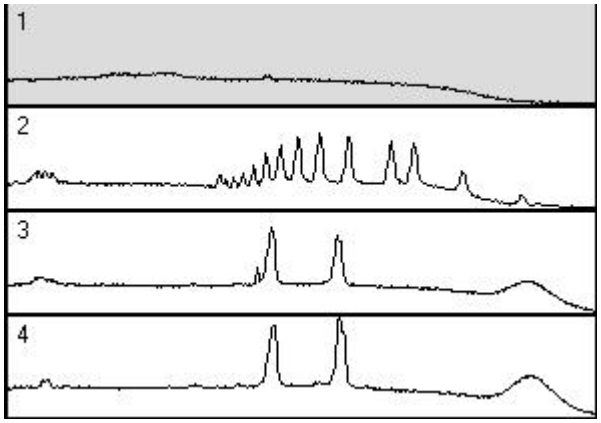
Data Table and Editing of Auto Lane

When the AUTO LANE button is clicked, the lanes are designated, bands found and the information is displayed in three windows: the upper window is the Active Graph, the lower left is the Graph Display and the lower right is the Data Window. The Lane Profile tab will also change to display the Auto Lane Editing functions. The data and display of Auto Lane is very similar to that given in the AutoGrid feature described above.

Graph Display

Graphs representing all the lanes on the template are shown in the lower left-hand quadrant.

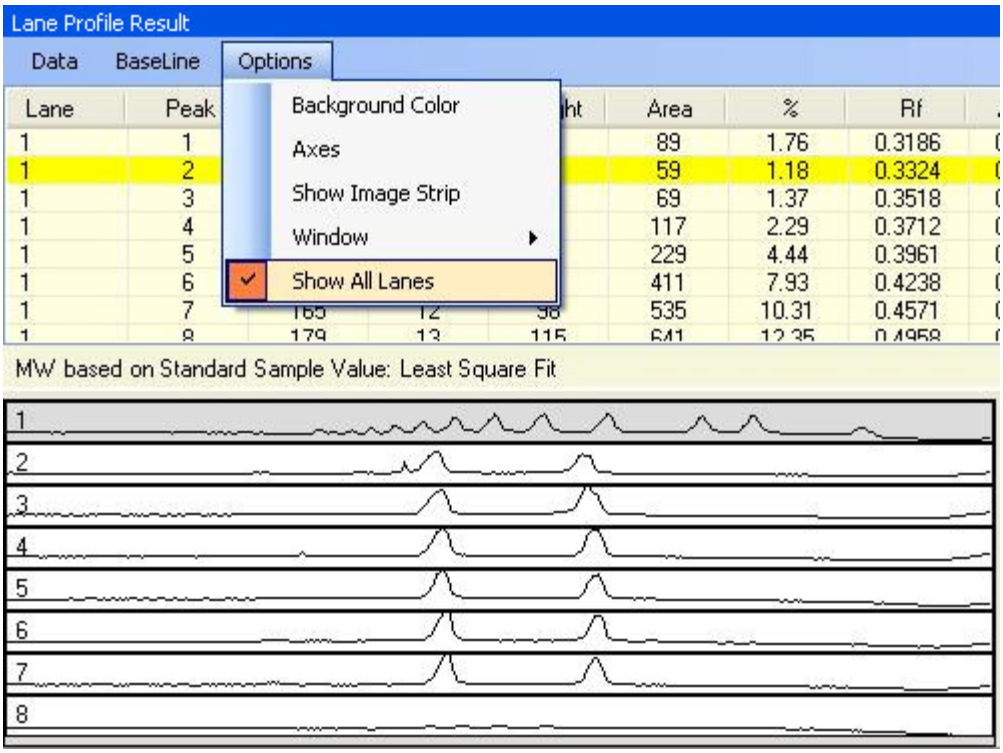
Lane 1 will be the default "active" lane when the scan is initially done. To select a different lane, simply click on its graph, and it will fill the Active Graph quadrant.



AlphaView uses the following color-coding:

- the active graph is shown in white
- graphs that have been viewed already are shown in yellow
- graphs that have not yet been viewed are shown in gray

All of the lanes can be displayed in the graph display quadrant by selecting "Show All Lanes" under the Options drop down menu in the Profile and Data window. By deselecting "Show All Lanes" only the first several lanes are shown providing more detail. A scroll bar allows for the other lanes to be viewed.

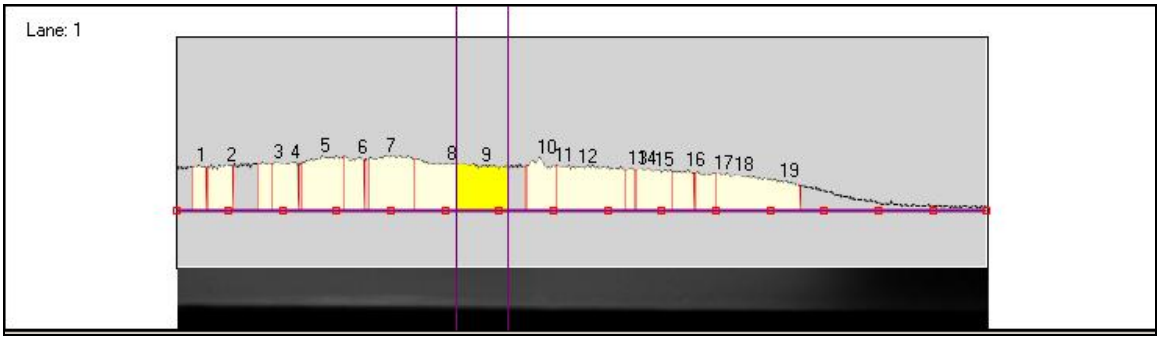


The Active Graph

The active graph is shown in the upper quadrant. The x-axis represents the distance (in pixels) from the top of the template. The y-axis represents the average pixel intensity across the width of the scan.

Visualization of the Active Graph

The drop down selections in the profile and data table (Data, BaseLine and Options) allow for the user to perform editing, visualization and outputting tasks pertaining to this window.



Data

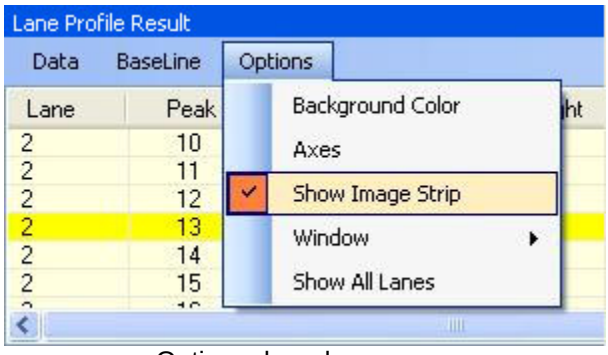
The data drop down menu allows for the user to superimpose different lanes onto the Active Window using the Overlay Control selection. (See Auto Grid Overlay Control above for detailed instructions.)

Baseline

Background subtraction can be preformed using this menu. (See Auto Grid Baseline Control described previously in this section for detailed instructions). Please note that background subtraction in AutoLane can have a dramatic effect on how many peaks are recognized as bands. Try changing the background subtraction to optimize band recognition.

Options

Background Color allows the user to select a variety of different background colors for the Active Graph. Axes allows the user to display the axes on the graph (x axis = pixel distance down the lane, y axis = pixel intensity in gray scale). Show Strip will display a strip of the lane being currently analyzed. Select Window will allow the user to choose which of the data table windows to display. Print will allow the user to print any or all of the profile and data table windows. Clipboard will allow the user to send any or all of the profile and data table windows to the clipboard for pasting into different programs (e.g. Excel™).



Options drop down menu

Editing Peak Boundaries

The boundaries of existing peaks can be readjusted to make the peaks wider or narrower. To adjust either peak boundary, click on the peak to select it. Next, point the cursor at the vertical boundary line, hold down the left mouse button and drag the line to the desired location. When it is in place, release the mouse button.

The data table is automatically updated to reflect any adjustments. Both the peak and the data in the data table will be blue to indicate that this is a user-defined (vs. automatically detected) peak.

The Data Window

Once a peak is defined, its integration data and associated information are displayed in a table located in the lower right quadrant of the screen. The data table is updated any time a peak is deleted or added, peak boundaries are redefined, or the background value is re-set.

Lane	Peak	Distance	Width	Height	Area	%	Rf	Adj. Rf
1	4	134	9	32	117	2.29	0.3712	0.3712
1	5	143	10	54	229	4.44	0.3961	0.3961
1	6	153	12	79	411	7.93	0.4238	0.4238
1	7	165	12	98	535	10.31	0.4571	0.4571
1	8	179	13	115	641	12.35	0.4958	0.4958
1	9	197	13	123	710	13.67	0.5457	0.5457
1	10	220	13	117	719	13.84	0.6094	0.6094
1	11	255	13	111	677	13.04	0.7064	0.7064
1	12	274	14	110	703	13.54	0.7590	0.7590
1	13	313	10	45	254	4.27	0.8670	0.8670
2	1	128	12	15	139	5.84	0.3546	0.3546
2	2	146	7	87	225	9.42	0.4044	0.4044

Example of an Auto Lane Data Table

PEAK is the number assigned to each peak on the graph beginning on the left and moving right.
DIST is the distance (in pixels) that each peak starts from the beginning of the line scan.
WIDTH and **HEIGHT** refer to the size of each peak.
AREA is the integrated area under each peak. This number reflects the intensity of each peak.
% is the percentage each peak contributes to the total density measured on the graph. (The sum of this column will be 100% for each lane.)

Auto Lane Editing Features

After the image has been evaluated for lanes and bands the following editing options will appear in the Lane Profile tab.

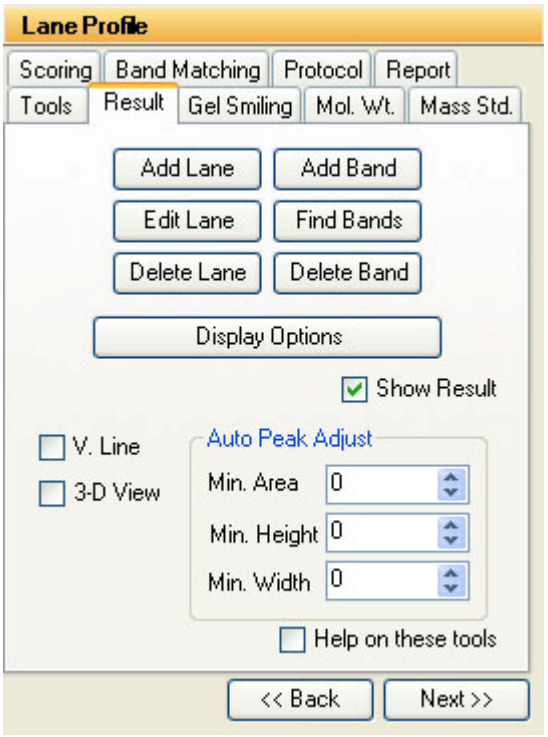


Figure 5.47 Auto Lane Editing Features

Add Lane
This feature allows an additional lane to be placed on the image. Once the button is selected the additional lane can be viewed and positioned on the image. A left mouse click will place the lane on the image. The data tables will readjust accordingly, and the bands within the lane calculated. As with most AlphaView features a right mouse click will reactivate the function and another lane will be ready to be added.

Edit Lanes
This feature allows for the horizontal manipulation of the center line in each of the found bands. The integrated area of each band will be altered, as the curve is re-figured to match the pixels surrounding the centroid mark.

Delete Lane
This selection will activate the cursor to delete a lane on the gel image. After selecting the button left mouse click on the lane in the image to delete. As with all AlphaView functions a right mouse click will reactivate the function for further lane deletions

Add Band
This selection will activate the cursor to add a band onto the gel image. After selecting the button left mouse click on the area in the gel in which a band should be added. As with all AlphaView functions a right mouse click will reactivate the function for further band additions.

Find Bands
This selection will re-detect the bands in the lanes based upon a new sensitivity setting 0-9. The higher the sensitivity the more bands the software will find.

Delete Bands
This selection will activate the cursor to delete a band in the gel image. After selecting the button, left mouse click on the area in the gel in which a band should be added. As with all AlphaView functions a right mouse click will reactivate the function for further band deletions.

Display Options
This feature allows the user to change the visual display of the lanes and bands on the analyzed image.

- Under Band Representation, the Cross selection designates bands with a cross symbol. The Box selection will display a 2 dimensional box around each of the bands.
- Lane Property gives the option of either showing the lane name by number (Numerical) or by letter (Character).
- Show Center Line selection displays the vertical line which runs through the center of each of the bands.
- Show Lane Bounds displays the perimeter of each of the lanes found in the gel.
- Show Band Properties turns the band property display on/off. The Band Property value to be displayed is selected under Band Property.
- Band Property allows the user to select what value to display as the bands property. The choices are Position (from top to bottom) in Numerals, Position (from top to bottom) in Characters, Rf, Adjusted Rf, or Molecular Weight.

Options

Gel Smiling Correction with Gel Smiling Tool

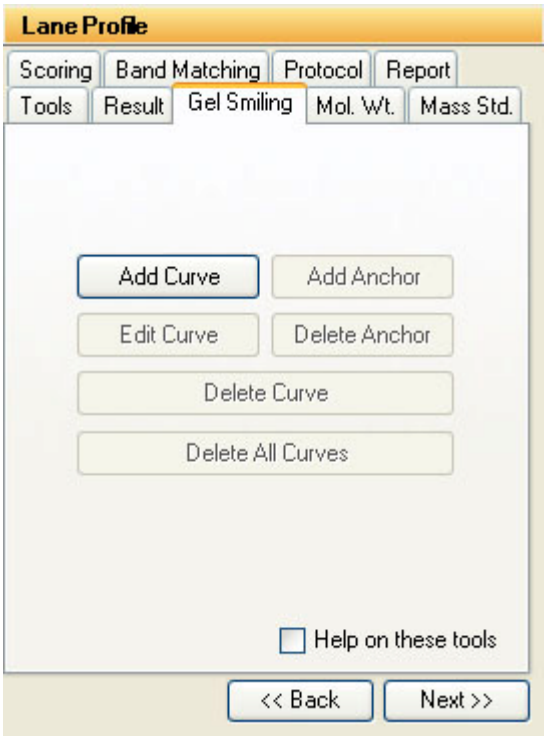
The Lane Profile Analysis module of the software contains the Gel Smiling tool which allows the user to adjust for gel smiling or curvature. Using this tool will increase the accuracy of Molecular Weight calculations and Automatic Band Matching. After the Gel Smiling have been applied a new column of data will be generated, Adjusted Rf. The Rf. values reported are relative values from 0 to 1 where 0 is the origin and 1 is the lane front without correction for gel smiling as commonly occurs in gel electrophoresis. The Adjusted Rf. (Adj. Rf.) is the corrected value.

Suggestions

The software calculates Adjusted Rf. based on the bands position relative to the nearest Gel Smiling Curve. When the band lies between two Gel Smiling curves with different curvatures a weighting is applied where greater weight is given to the nearest Gel Smiling Curve. For the most accurate calculations enough curves should be added to properly mimic the curvature of the sample. We also suggest placing at least one Gel Smiling Curve above the top most band and below the bottom most band. If these curves are left out the software will use the lane origin and front as the first and last curves. These are assumed to be straight.

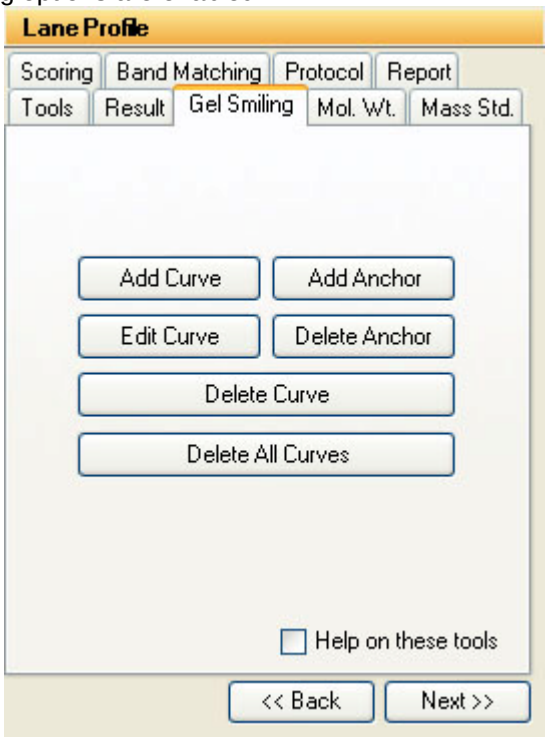
Applying Gel Smiling Curves

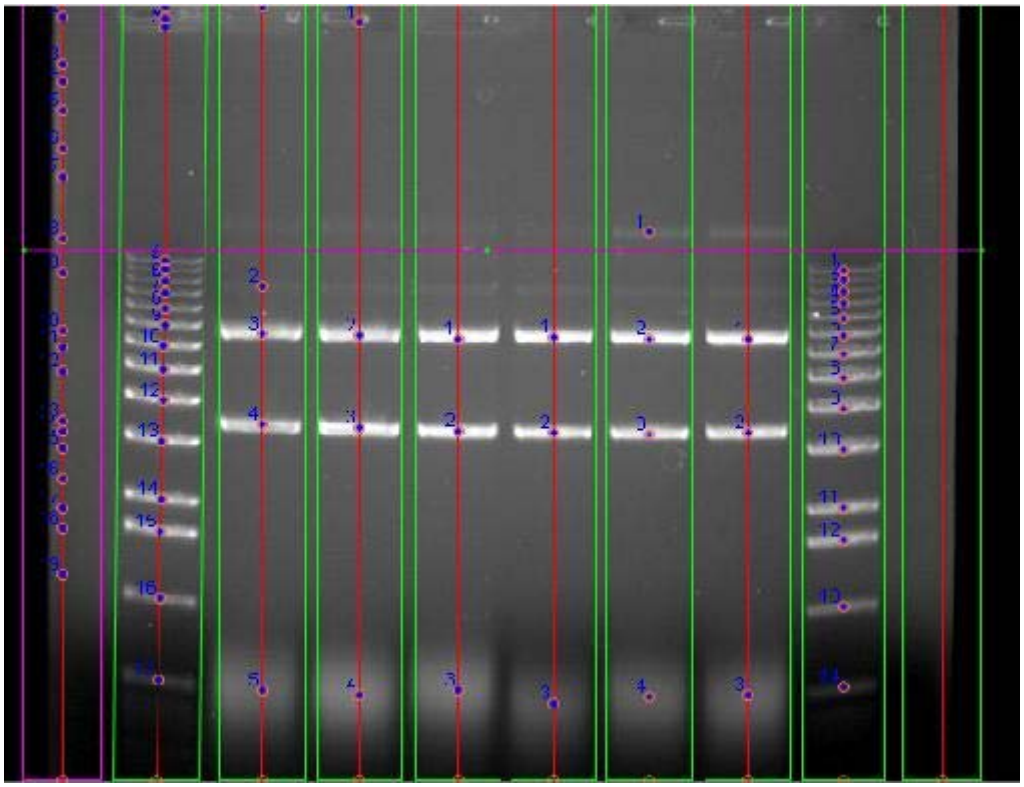
After identifying and editing lanes, the Gel Smiling tool can be accessed.



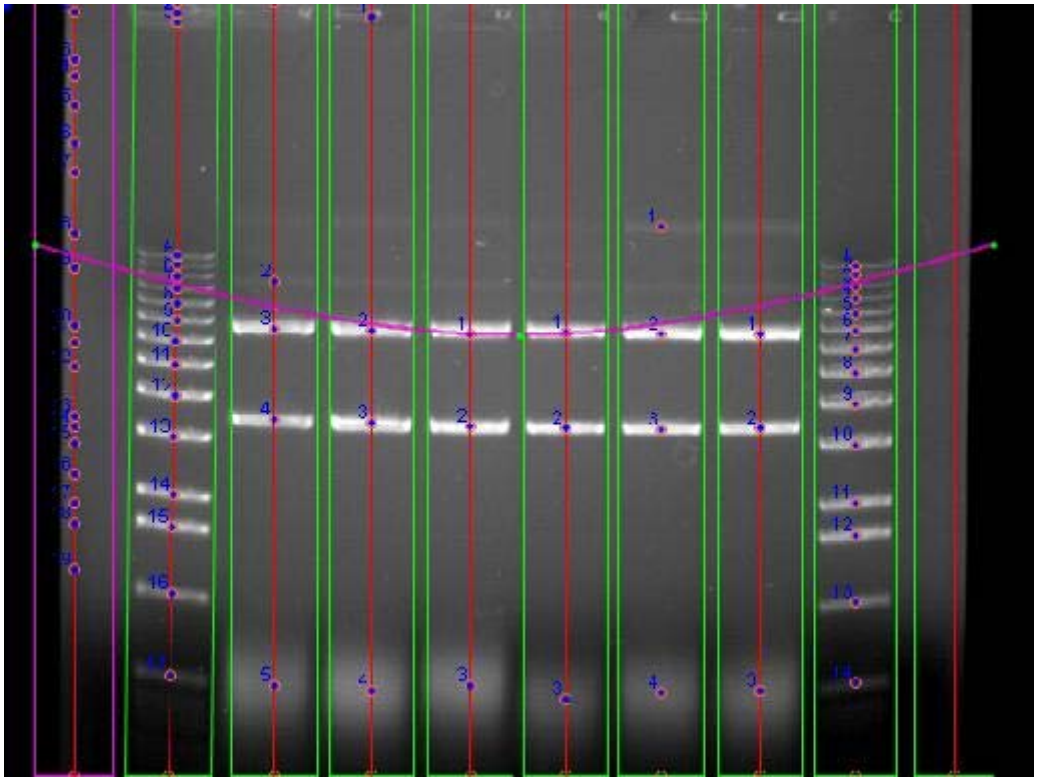
Add Curve

Click Add Curve button and then click on the image to apply the curve. Clicking within the lane boundaries will produce a three point curve with an anchor point each on the boundary of the first and last lanes and one at the position of the mouse click. Once a curve has been applied, following other Gel Smiling options are enabled.





Adjust the curve by dragging the yellow anchor points.



Adding/Removing Anchor Points

Anchor points can be added by first Selecting the Add Anchor option from UI and then clicking on or near any Gel Smiling Curve. When the cursor changes to a cross ┼, it will add an anchor point to the nearest curve by clicking the left mouse button once.
Additionally, anchor points can be deleted by first selecting the Delete Anchor option from UI and then clicking once on the anchor will remove anchor point.

Remove Curve

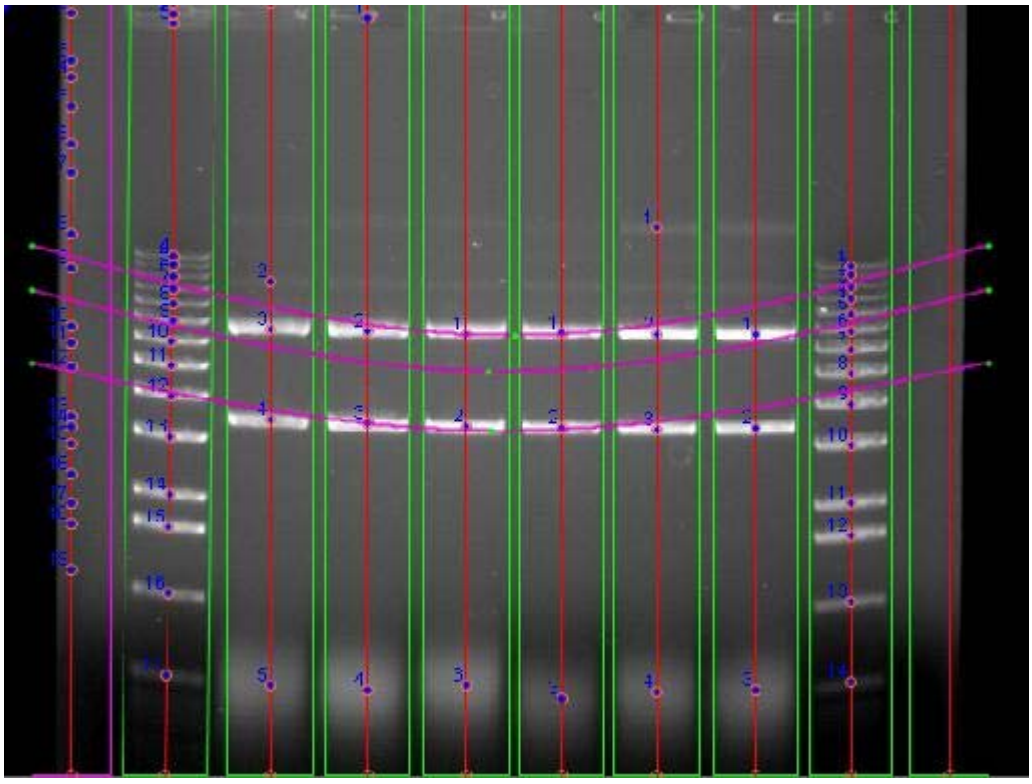
A Curve can be deleted by first selecting the Delete Curve option from UI and then clicking on the appropriate curve will delete the curve.

Adjusting Curve using Anchor Points

Click Edit Curve to adjust anchor points by clicking and then dragging the anchor point from one location to the next.

Additional Gel Smiling Curves

Apply enough Curves to properly mimic the curvature of your sample. The more curves applied, the more accurate calculations will be for molecular weight and band matching. After the initial curve is applied the software will attempt to mimic the curvature of the surrounding curves when new curves are applied. This results in less editing of the additional curves.



Band Matching, Similarity Matrix, and Dendrograms

AlphaView provides features for matching bands between lanes. The resultant data can then be used to generate similarity matrices and/or dendrograms. Band matching features are accessible under the Analysis menu within Lane Profile's AutoLane feature.

Prerequisites for Band Matching

Prior to performing band matching, a number of steps must be followed.

1. Lanes and Bands must be detected and edited through the AutoLane feature.
2. If Molecular Weights are to be calculated, molecular weight markers must be loaded prior to band matching. Refer to the section within Lane Profile on loading molecular weight markers.
3. (Optional) A new Gel Smiling tool has been added to AlphaView. This tool allows the user to adjust the software's calculations for M.W., Adj. Rf, and Band Matching by drawing the shape of the gel smile or curvature. Applying the Gel Smiling tool will aid in the accuracy of automatically matching bands and will result in less user edits afterwards. Refer to the section on Gel Smiling tool for further instructions.

Matching Bands

Band Matching dialog window is accessible through the Analysis menu on AutoLane's Profile and Data window. Select *Band Matching* from the *Analysis* menu.

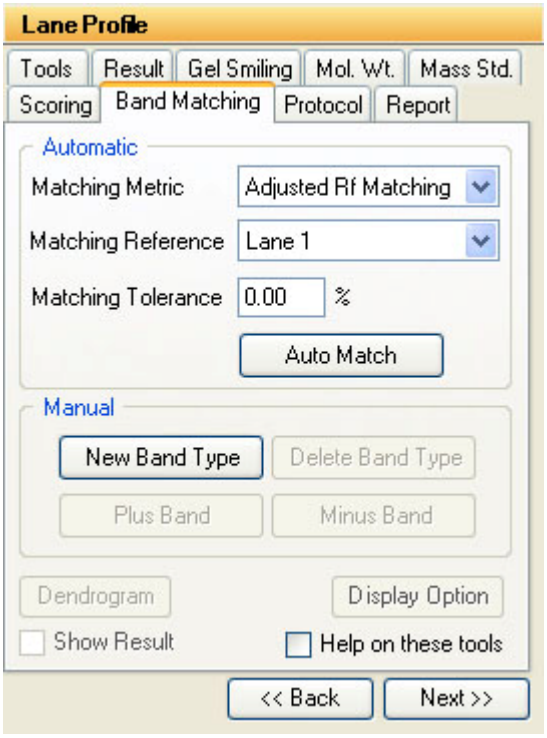


Figure 5.48 Band Matching Dialog window

Automatic versus Manual Band Matching

Functionality is provided for both Automatic and Manual band matching. Each will be explained in detail. Note that these are not exclusive. After Automatic Band Matching has been performed the user is able to use the Manual matching features to edit or correct the automatic findings.

Automatic Band Matching

Matching Metric

Select either Adjusted Rf Matching or Generic Rf Matching as the matching metric.

Adjusted Rf Matching: When Adjusted Rf Matching is selected the software will match bands based on the data values in the Adjusted Rf column of the Profile and Data window. These values result from the gel smile/curvature rather than the absolute vertical Rf value. Adjusted Rf is calculated based on the addition of M.W. markers and/or the Rf Curve lines which serve to correct the Generic Rf values for gel smiling.

Generic Rf Matching: When Generic Rf Matching is selected the software will match bands based on the data values in the Rf column of the Profile and Data window. These values are the relative Rf calculated vertically where the origin of the lane is set to zero and the lane front is set to 1. Gel smiling or curvature is not adjusted for in this selection.

Matching Reference

Select a lane to serve as the matching reference. It is advised that you choose a lane that either contains most of the bands of interest or spans the range of bands of interest while also containing many of the bands of interest. This will reduce the amount of effort required for post match edits.

Matching Tolerance

Enter a value to be used as the matching tolerance. The tolerance is converted into the amount of displacement two bands may have and still be matched by the software. The larger the tolerance value the larger the range the software will use for matching. The valid range is 0-10%. A value of 1-5% typically works best. A lower percentage works well for tightly spaced bands.

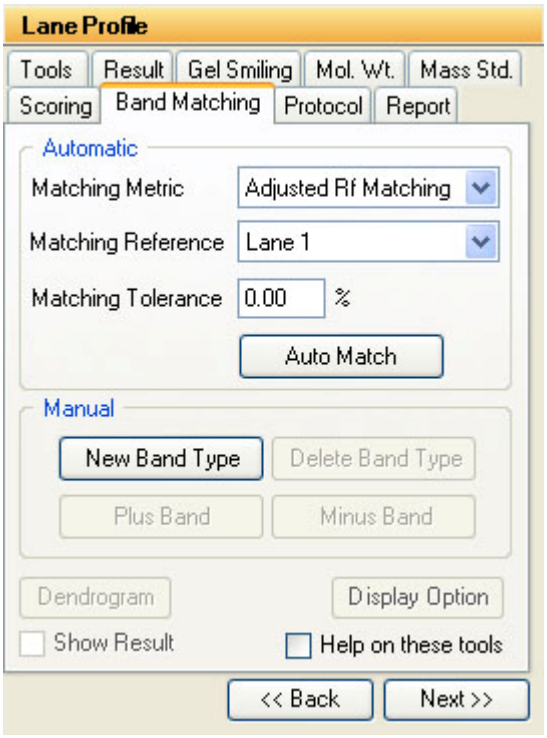
Auto Match

After selecting a matching metric, reference, and tolerance click the Auto Match button for the software to perform auto matching. When matching has been completed the Display Results and Generate Dendrogram buttons will become active. Refer to the appropriate sections below for a description of Similarity Results and Dendrogram creation.

Editing Match Results

Following Automatic band matching the user may need to manually edit or correct some matches. The tools under Manual Matching can be used for this purpose. Refer to the section below on Manual Matching for a description of each tool.

Manual Band Matching



Band Matching Dialog window

Manual band matching tools are useful for quickly matching lanes with only a few bands or for manual edits and corrections to Automatic Band Matching results.

New Band Type

Select the New Band Type tool by clicking the New Band Type button. A Band Type is a band with a unique Rf. or Adj. Rf. Value. Each band within a lane will be part of a band type and no two bands within a lane can be of the same band type.
After selecting the New Band Type tool, click on a band to draw a band type line. Repeat this process of selecting the tool and clicking a band until a band for each unique band type has been selected.

Deleting a Band Type

To delete a band type, select the Delete Band Type button and click on a band type line.

Adding Bands to a Band Type

Select the Plus Band tool by clicking the Plus Band button. Click on a band type line to select it, the line will be highlighted in yellow. Now click on a band that belongs to this band type. Repeat this process for all bands belonging to this band type. Then repeat the process for each band type until all bands have been identified as belonging to a band type.

Removing Bands from a Band Type

Select the Minus Band tool by clicking on the Minus Band button. Click on a band type line to select it, the line will be highlighted in yellow. Now click on the band that you would like to remove from the selected band type.

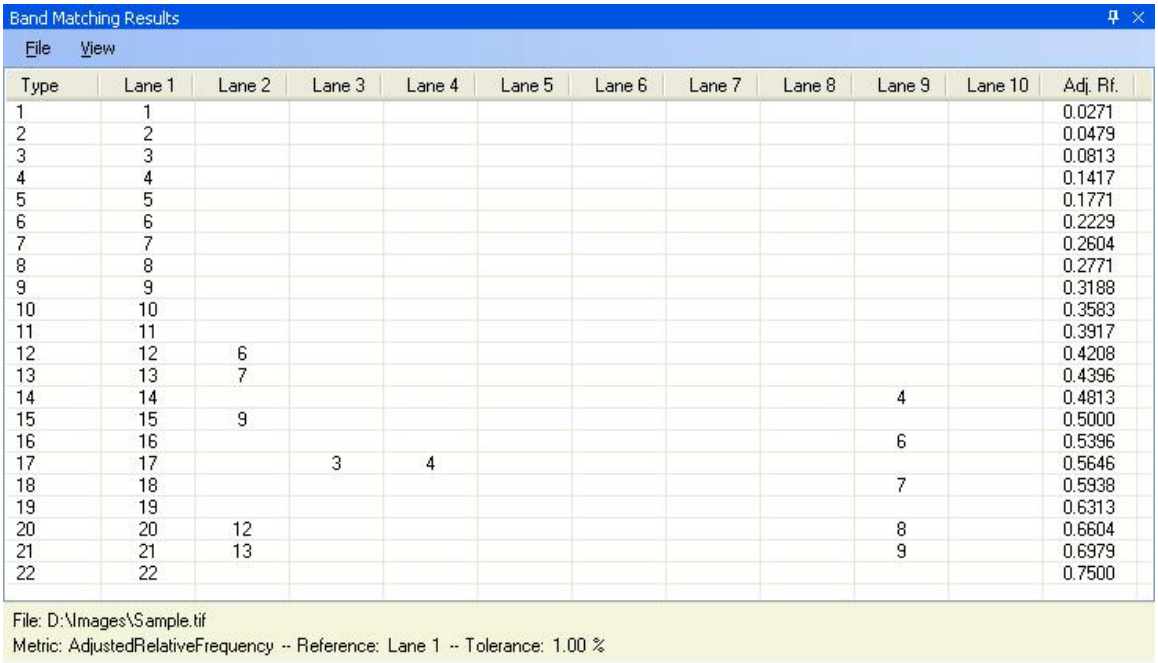
Undo/Redo

The Undo and Redo buttons will undo and redo the last action performed respectively.

Band Matching Results

Display Results

After bands have been matched a table is generated with the matching results. Click the Display Results button to view the matching results.



Type	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10	Adj. Rf.
1	1										0.0271
2	2										0.0479
3	3										0.0813
4	4										0.1417
5	5										0.1771
6	6										0.2229
7	7										0.2604
8	8										0.2771
9	9										0.3188
10	10										0.3583
11	11										0.3917
12	12	6									0.4208
13	13	7									0.4396
14	14								4		0.4813
15	15	9									0.5000
16	16								6		0.5396
17	17		3	4							0.5646
18	18								7		0.5938
19	19										0.6313
20	20	12							8		0.6604
21	21	13							9		0.6979
22	22										0.7500

Figure 5.49 Band Matching Results window displaying the similarity matrix

Viewing Result Tables

The match results can be viewed in one of four configurations. Select the configuration desired under the *View* menu. In each view Band Type is identified in the left most column. Band numbers appear in their Rf. Position within each lane. Lane numbers are identified in the first row. The file location, matching metric, matching reference, and matching tolerance are displayed below the tables.

Mixed Table: This display will show you both matched and unmatched bands in one table.
Separate Tables: This display will show you matched and unmatched bands in separate tables.
Matched Table: This display will show you only the results of matched bands.
Unmatched Table: This display will show you only the results of unmatched bands.

Exporting and Printing Band Matching Results

Print and Export are selectable under the *File* menu. Select Print to send the report to a local or network printer. Select *Export* to export the results to the clipboard or to file. When exporting to a file there are two file types that can be saved, standard text file (*.txt) or a comma separated value file (*.csv). When exporting to Excel, use the *.csv file type to skip the Excel import wizard or use export to clipboard followed by Paste in Excel.

Dendrogram and Similarity Matrix Generation

After bands have been matched dendrograms and similarity matrices can be generated from the data. Click the Generate Dendrograms button to view these.

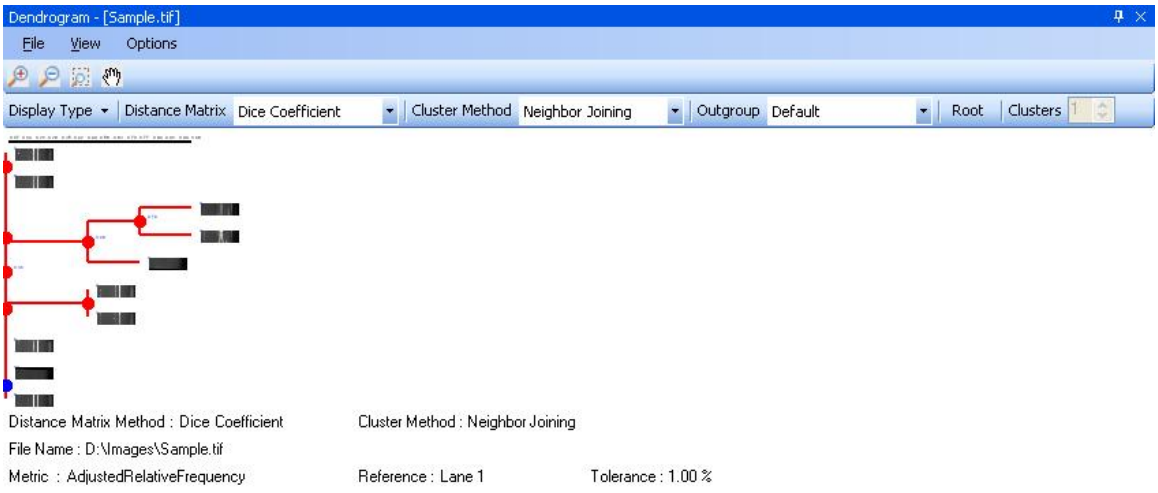
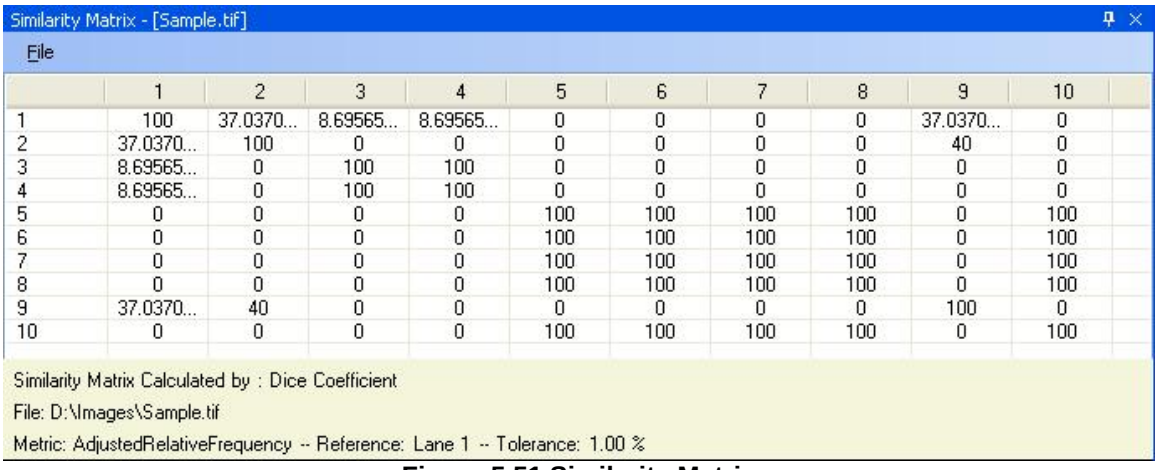


Figure 5.50 Dendrogram window

Dendrogram window where Dendrograms are generated and Similarity Matrices are accessed.

Similarity Matrix

Select a method from the *Distance* Matrix pull down for calculating similarity. The choices are Dice Coefficient, Jaccard Coefficient, Pearson Coefficient, and Frequency Similarity. These are standard statistical algorithms, references can be found in most statistical text books.
Select the *Display Matrix* option from the *Options* to display the Similarity Matrix.



	1	2	3	4	5	6	7	8	9	10
1	100	37.0370...	8.69565...	8.69565...	0	0	0	0	37.0370...	0
2	37.0370...	100	0	0	0	0	0	0	40	0
3	8.69565...	0	100	100	0	0	0	0	0	0
4	8.69565...	0	100	100	0	0	0	0	0	0
5	0	0	0	0	100	100	100	100	0	100
6	0	0	0	0	100	100	100	100	0	100
7	0	0	0	0	100	100	100	100	0	100
8	0	0	0	0	100	100	100	100	0	100
9	37.0370...	40	0	0	0	0	0	0	100	0
10	0	0	0	0	100	100	100	100	0	100

Figure 5.51 Similarity Matrix

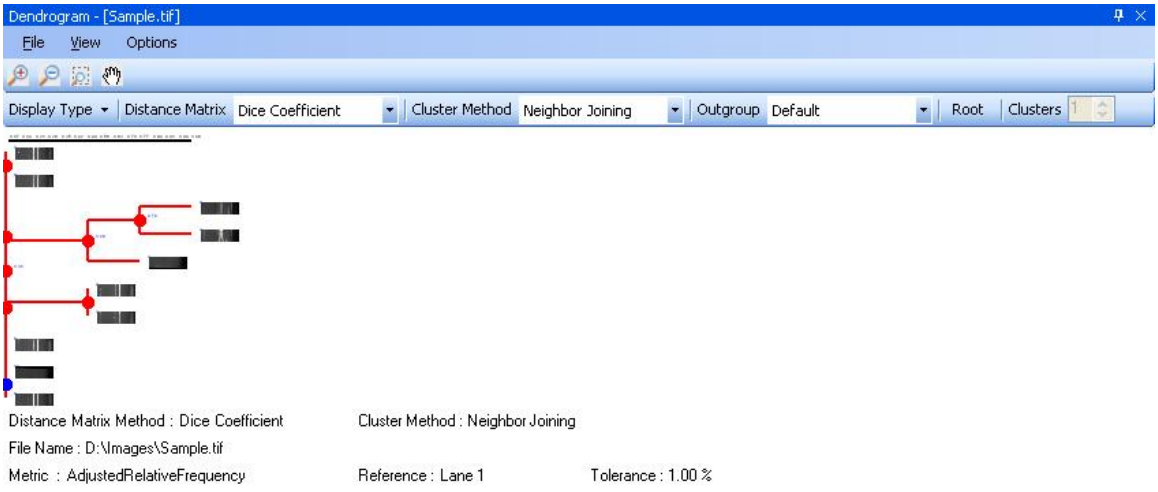
The similarity matrix is a graphical display of the similarity between lanes.

Exporting and Printing the Similarity Matrix

Print and Export are selectable under the *File* menu. Select Print to send the report to a local or network printer. Select *Export* to export the results to the clipboard or to file. When exporting to a file there are two file types that can be saved, standard text file (*.txt) or a comma separated value file (*.csv). When exporting to Excel, use the *.csv file type to skip the Excel import wizard or use export to clipboard followed by Paste in Excel.

Dendrograms

The dendrogram window is accessed by clicking the Generate Dendrograms button in the Band Matching dialog.



Distance Matrix

Select a method from the *Distance* Matrix pull down for calculating similarity. The choices are Dice Coefficient, Jaccard Coefficient, Pearson Coefficient, and Frequency Similarity. These are standard statistical algorithms, references can be found in most statistical text books.

Cluster Method

Select a method for calculating the clustering from the *Cluster Method* pull down. The choices are Neighbor Joining, UPGMA, WPGMA, Single Linkage, Complete Linkage, Ward, Median, and Centroid. . These are standard statistical algorithms, references can be found in most statistical text books.

Clusters

Select the number of clusters you would like displayed in the dendrogram. The values are 1 to maximum number of lanes in the image. This option is disabled when Neighbor Joining is chosen for the cluster method since the method determines the number of clusters.

OutGroup

When Neighbor Joining is chosen for the cluster method the OutGroup option is enabled allowing the user the ability to set the number of Out Groups to display.

Exporting and Printing the Dendrogram

Print and Export are selectable under the *File* menu. Select Print to send the report to a local or network printer. Select *Export* to export the results to the clipboard or to file. When exporting to a file there are two file types that can be saved, standard text file (*.txt) or a comma separated value file (*.csv). When exporting to Excel, use the *.csv file type to skip the Excel import wizard or use export to clipboard followed by Paste in Excel.

Display Type

Under the *View* menu the user may select to view the dendrogram in the Phylogram or Cladogram format.

Collapse/Expand

The Collapse/Expand tool allows the user to collapse or expand the dendrogram by clicking on a red intersection marker.

Zoom In/Out Tools 

The zoom in and out tools allow zoom control over the dendrogram window.

Zoom with Selection Tool 

The zoom with selection tool allows the user to zoom an area of interest within the dendrogram defined by mouse.

Grab Tool 

The grab tool allows the user to navigate the entire dendrogram in a zoomed display by clicking and dragging to window.

Common Features

Default analysis tool has three common features namely, Protocol, Report, and Export Results.

Protocol

Figure 5.52 Protocol Tab

The **Protocol** tab is used to save and load protocols for use on replicate blots.

Saving a protocol differs from saving an analysis in that protocols may be used on images other than the original image while a saved analysis is available for loading only on the original image.

Note that an analysis may be saved at anytime by using the file drop down menu.

Figure 5.53 Saving an analysis

A protocol or analysis contains all band and background regions created with the loading controls, band controls and standard curve settings used. A protocol or analysis may be saved at any point in the analysis workflow.

Report

Using Report Tab, user can generate reports based on analysis data. Reports offers different options to customize the report, which can be saved on file as an Html Format (*.html) or Rich Text Format (*.rtf). User can also take print of report.

Reports also allows user to save report settings/configuration as template or can modify existing templates.

Formatting

Formatting tab allows user to format reports before saving or sending it to printer.

User can create new report or select existing report template to create a report using the drop down list. If no report template is defined/saved, it will be disabled.
Choose image that will be part of report with the image size. 1/4 is the default image size.
Also choose different information (Data sheet, Graph, Acquisition information) that will be part of report.

Report settings/configuration can be saved using Save button, in case of new or Save as in case of modifying an existing report template.

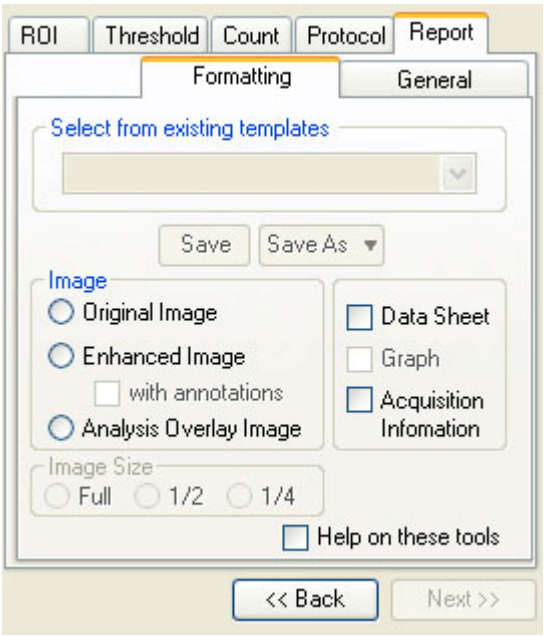


Figure 5.54 Report – Formatting Tab

General

General tab allows user to give report a title, enter analysis notes / comments or give user name.

Clicking Generate Report will create a report based on report settings/configuration.

Figure 5.55 Report – General Tab

Export Results

Once the analysis has completed then there is option to export the result. Either the results can print directly to a printer, or export the results as ASCII data for direct importation into Excel or other spreadsheet programs

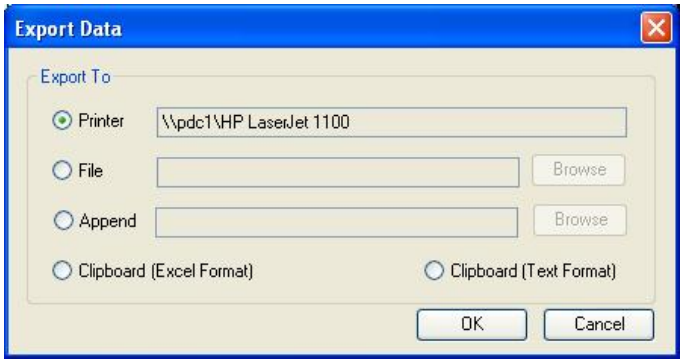


Figure 5.56 Common Export Dialog Box

Sending Data to a Printer

To send the data directly to the Video Printer or Default Printer, click on the Export button and click in the circle next to appropriate printer.

Sending Data to a File

If you would like to take the data from the system and import it into another computer, the data can be saved to a diskette or network which will allow you to open the data file on a separate workstation connected to the network.

Click on the Export button and then on the File option. Specify the path and file name to send the data file. The data is saved as an ASCII file and can be imported into most spreadsheet programs. ASCII is a very common file format output option for numerical data.

Appending Data to a Same File

Export dialog also allows you to append data to an existing exported file.
Note: This feature is applicable to AutoCount and Array analysis modules.

Sending Data to a Spreadsheet Program

To send the data results to Excel or other spreadsheet programs, click on the Export button, click on the clipboard option, then click on OK. If you have Excel or another spreadsheet program loaded on the system and running in the background, you can simply press the ALT and TAB keys simultaneously to move into the spreadsheet program. You can then import the data directly to the desired spreadsheet from clipboard.
Note: The spreadsheet program must be installed on the computer in order to export the data to that program.

When the appropriate export source has been selected, click on the OK button to send the data.

Exporting Quantitative Data Lane Profile

The exporting of Lane Profile is similar to what has been defined above with some extra feature.

The Lane Profile has following window to export data.

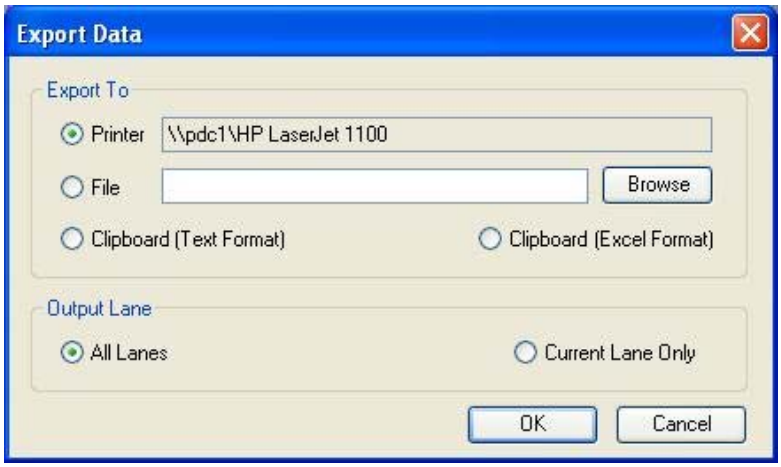


Figure 5.57 Lane Profile Export Dialog Box

Output Lane

This option allows user to export what type of data to be exported. Selecting All Lanes will export all lanes data or selecting Current Lane will only export current lane data.

To send the data, click on the OK button.

Additional Analysis Tools

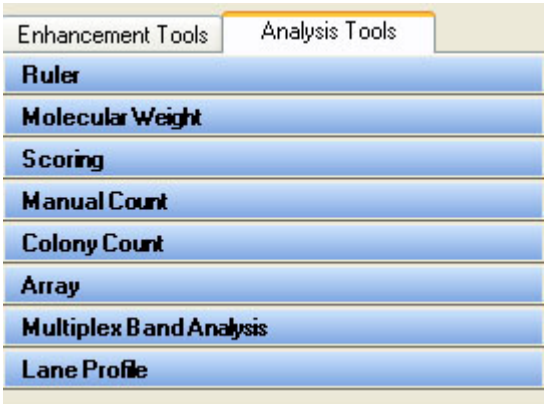
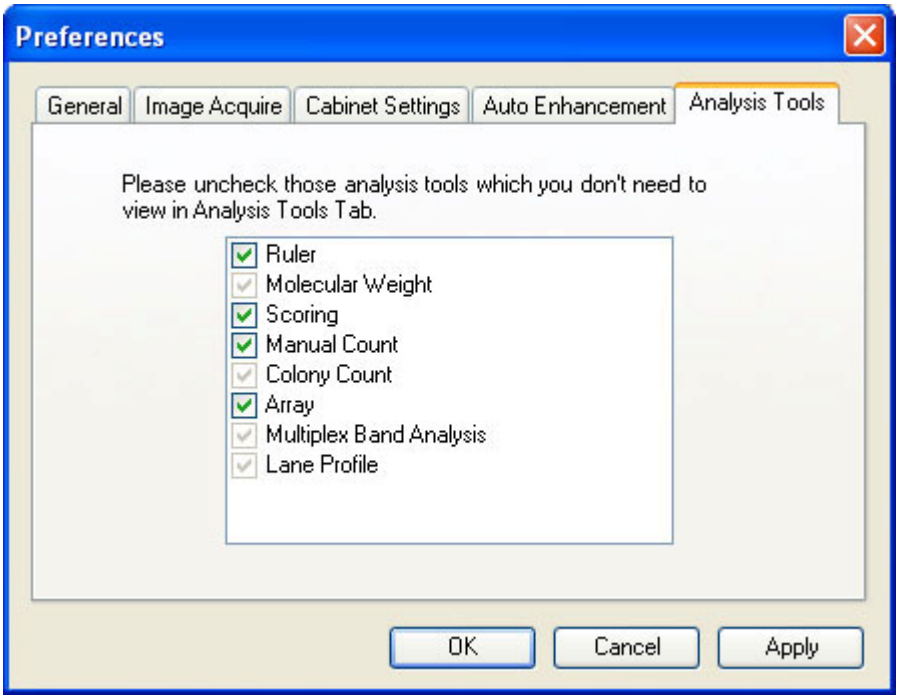


Figure 5.58 Additional Analysis Tools in the ToolBox

Additional image analysis tools can be activated to show up under the tool box as shown here. To activate these tools:

1. Go to Setup> Preferences > Analysis tools tab.
2. Select tools to show.



This tool set allows the user to perform ruler measurements, gel scoring, manual counting of colonies and cells, and perform high density array analysis.

The Ruler Function

Introduction

The Ruler function allows the user to create a scale based on a standard and to measure distances from a set origin to a chosen location.

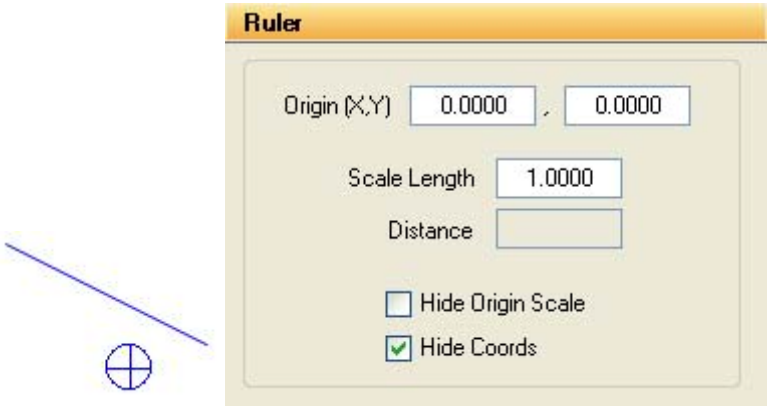


Figure 5.59 The Ruler Tools and Ruler Toolbox

Using the Ruler Function

To access the Ruler function, click on ToolBox, Analysis Tools and then on RULER. A line with a box at each end and a circle with a cross in it will appear on the screen.

Before any measurements can be obtained, a standard scale must be established. The scale that is created will serve as the standard for all measurements. Therefore, it is advisable to draw the scale line according to some standard measurement within the image (such as a ruler).

Using the mouse, click and drag one end of the line and then the other end into position. Once the line is in position, click in the SCALE LENGTH box and type the length of the standard. **Press <enter> to save this value**.

Distance is measured from the origin (designated by the circle with a cross in it) to the mouse location. Place the origin marker appropriately, and then move the mouse. The distance will be constantly updated in the DISTANCE box.

To hide the scale and origin, click on the HIDE ORIGIN SCALE checkbox.

Cartesian Coordinates

The Ruler function can also be used to create a Cartesian coordinate system. The origin icon has default values (0.000, 0.000). These can be changed by clicking on the ORIGIN box, typing in new values, and clicking <enter>.

Once the origin is placed on the screen in its desired location, the relative coordinates of the mouse at any location are shown at the right-hand side of the screen. In the example above, the cursor is 0.747 inches to the right of the origin and 1.071 inches below. To hide the coordinates, click on the HIDE COORDINATES checkbox.

The Scoring Function

The Scoring function is designed for gene expression applications to check for presence or absence of specific samples. The scoring function can be used for a quick manual identification of several different sample types. The software allows for up to three different scores on a gel, blot, or microtiter plate.

To score an image, click on the SCORING button in Toolbox 4. Define the appropriate number of rows and columns of your image by using the appropriate controls. You can also define the amount of spacing between your rows and columns to compensate for any variations between lanes or wells.

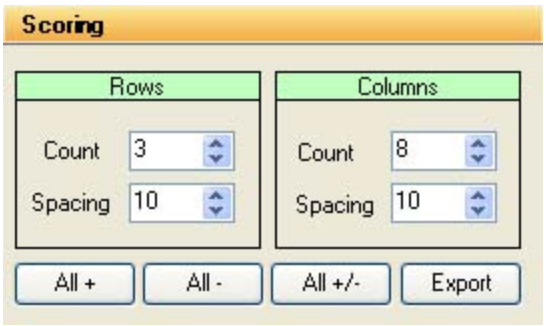
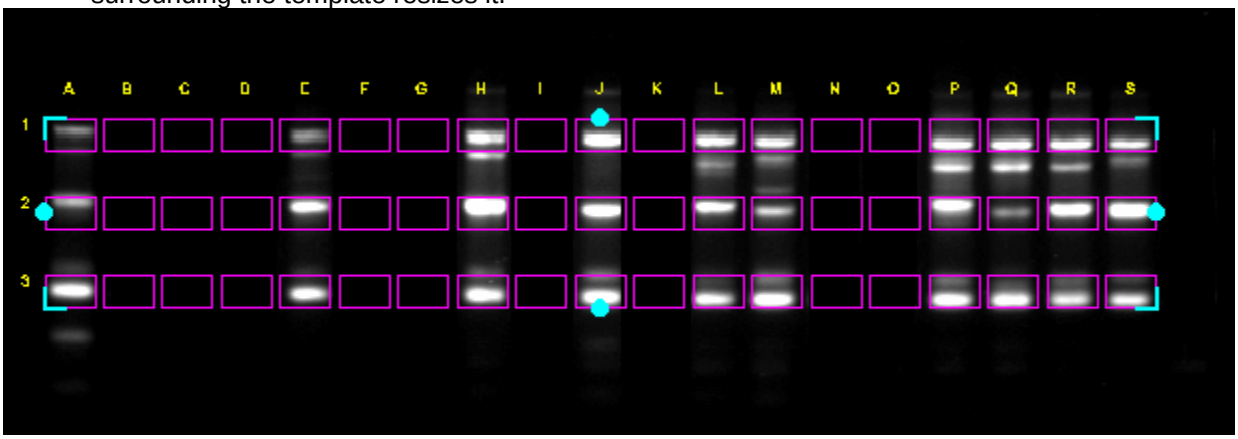


Figure 5.60 Scoring controls

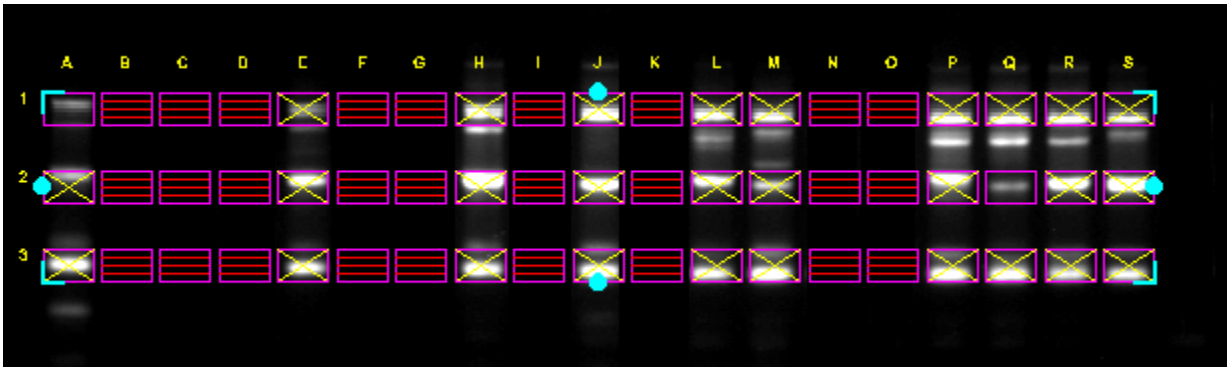
Once you have selected the number of rows and columns to be displayed, you may adjust the template to fit your image. Use the left button on the mouse to click, hold, and drag the outside borders of the template so they frame the lanes or wells to be scored. Clicking, holding, and dragging on or within any border moves the entire template. Clicking on the blue corners surrounding the template resizes it.



Scoring image with template positioned

Scoring the Sample

Three different scoring options are available: score with a positive, negative, or positive / negative. The software will display an “X” if you have a positive sample or three “–“ signs arranged in a vertical fashion if you score with a negative. Nothing will appear if you score with a positive/negative. You may use the right and left mouse buttons to determine different scores. The right mouse button will score the sample with an “X”. The left mouse button will score the sample with three “-“ signs vertically arranged.



]Scoring for positives (+), negatives (-), and positive / negative (nothing)

For faster scoring, you may look at the entire image and visually determine whether the samples are primarily positive, negative, or positive / negative. By clicking on the All + , All -, or All +/- boxes, all of the samples on the image will have either an “X”, three “-”, or nothing depending on the button you clicked.

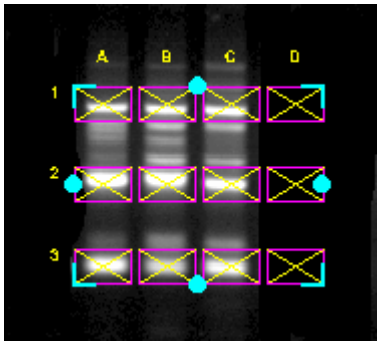


Image after All + button pressed

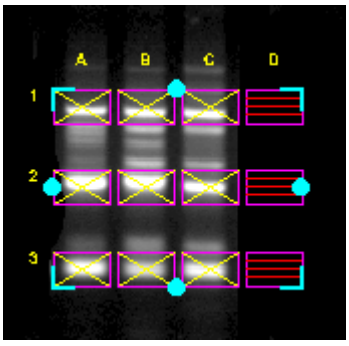


Image after All + button pressed and individual sample scoring -

Output and scoring options

Manual Count

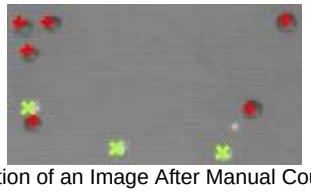
Click on MANUAL COUNT. The following sets of tools will be displayed in the lower left of the screen. Some samples are extremely difficult to count because of their shape, size, or lack of intensity variation from the background. To accurately count these samples, we have developed the manual counting feature within our software. To manually count the objects in an image, click on MANUAL COUNT in Tool Box, Analysis Tools. You may use either a green x or a red +. If you are only counting one type of sample, you may simply use the x which is the default to begin counting in the software.

Figure 5.61 Manual Count Tools

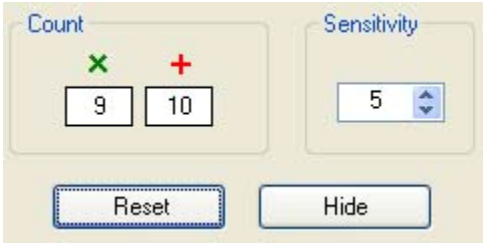
Placing Markers to Count

Point the cursor at an object to be counted and click the left mouse button. An "x" is placed over the object, ensuring that the same object will not be mistakenly counted twice. In the toolbox, the number in the COUNT box automatically increases by 1. Click on all of the objects of this type or color to be counted.

To count objects of another type or color (such as white vs. blue colonies in a β-gal assay), use right mouse button to click on each object as above.



Section of an Image After Manual Counting



Corresponding Data

Erasing and Hiding Count Markers

To erase a marker that has been manually placed, click on the marker on the image. It will disappear and the count will be reduced by one.

The Sensitivity indicator determines how close the cursor must be to a marker in order to delete it. Higher numbers allow the user to click further away from the marker to delete it; however, if Sensitivity is set too high, it will be impossible to place markers on spots that are close together.

Hiding Your Count Markers from the Screen

The software will record all counts as you continue clicking on your objects. If the counting becomes confusing, you may click on the HIDE button to hide off of the x's and +'s that were used to count your objects on the screen. If you wish to make a print of your image hiding the count, make sure that the HIDE button has been pressed to high your count markers. You can then go to Tool Box, Enhancement Tools, Annotations, and label the image with the appropriate. Click on the PRINT button to obtain a hardcopy.

Erasing the Count Markers and Data

Once you have finished counting your objects and have obtained the data you need, you may clear the count by clicking on the ZERO button. This will erase all of the count markers from the screen and zero the count data to allow you to begin counting again with the same image or another image that you have acquired.

Analyzing Arrays

The ARRAY analysis tools (found in the ToolBox, Analysis Tools) make it easy to measure the relative gray levels of objects in a uniformly spaced array, such as microtiter plate wells or dot blots.

These tools allow spot number, orientation and size to be specified. For microtiter plates, two options are available: measuring the density within a circle (well), or measuring a central spot and a surrounding torus (halo) separately.

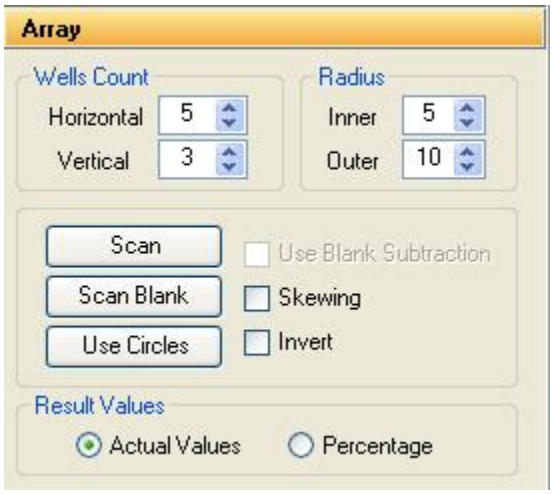


Figure 5.62 ARRAY Toolbox

Once these parameters have been set, a template can be saved in a default file and recalled at a later time. (See Section 3.5 for details on saving defaults.)

Setting up an ARRAY Template

To access the ARRAY tools, open ToolBox, Analysis Tools and click on the ARRAY button. A template is displayed on the image, depicting the layout of the objects to be measured. Up to 10,000 objects in a 100x100 array can be measured. (The default is an array of 96 circles, arranged in 8 rows of 12.)

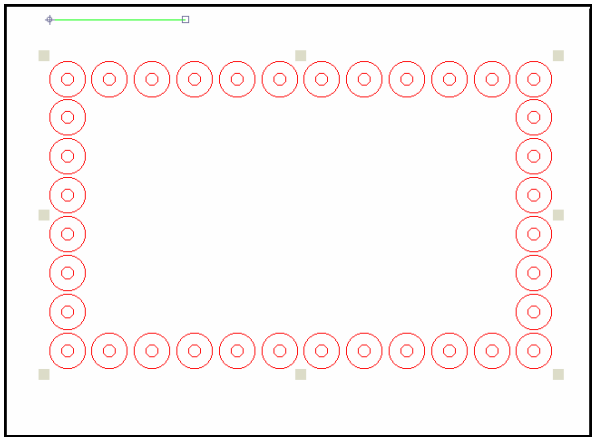
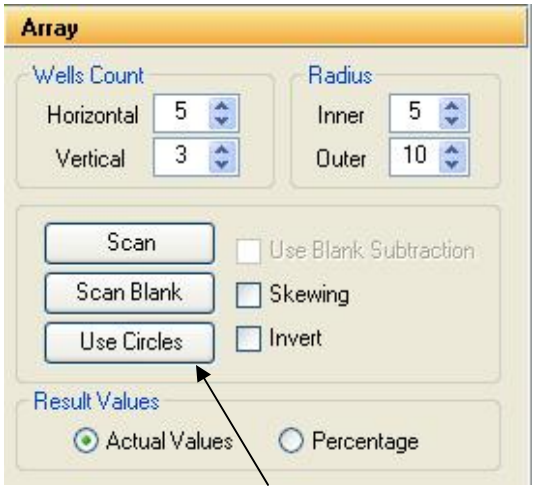


Figure 5.63 ARRAY Template

The tool box work area displays a number of buttons and controls for specifying the number of objects, along with their orientation and sizes.

Analyze arrays with Circles or Squares



Switch between "Use Circle" objects and "Use Square" objects

The controls labeled Hori. Wells and Ver. Wells let the number of horizontal and vertical objects be set (to a maximum of 100 and 100, respectively). The controls labeled Center and Outer let the size of the center and outer circles be set. Click on an arrow to increase or decrease the number of objects per row or column, or the size of the objects. As the number and size are adjusted, the template displayed over the image is updated to reflect the changes.

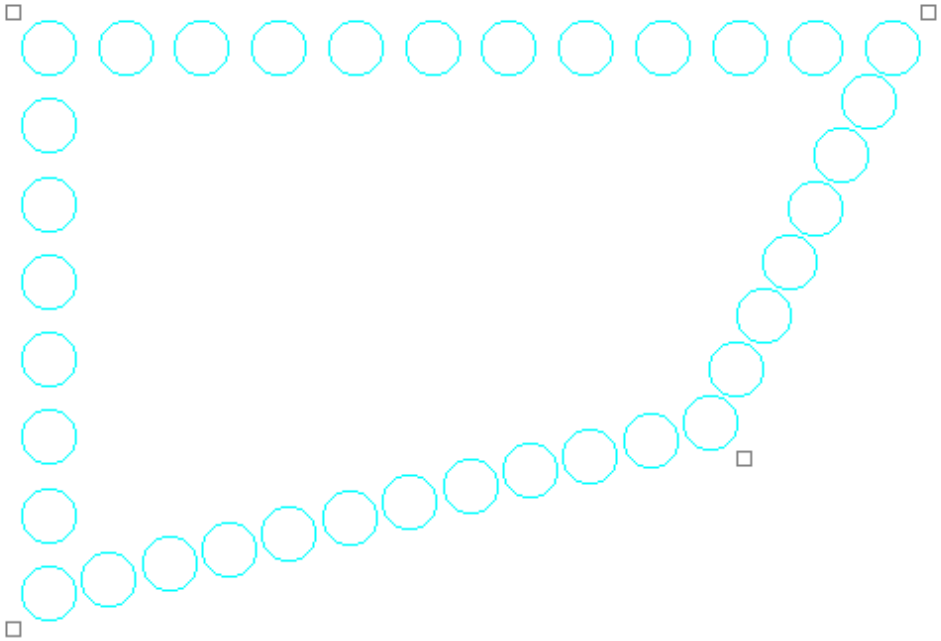
Aligning the Template

Once the number of objects has been set, adjust the placement of the template.

If the image of the sample is not parallel with the template, the template can be realigned by adjusting the green line so it is parallel to the top edge of the sample. Place the cursor on the box at either end of the line. Hold the left mouse button down and drag the line until it is parallel to the image.

Next, adjust the placement of the template so the objects coincide with the areas of interest on the image. Move the cursor to any of the square "handles" on the outside of the template. Hold down the left mouse button and drag the handles until the template is properly positioned.

If the image is not perfectly rectangular, the template can be skewed by clicking the Skewing checkbox.



A Skewed Template

Specifying the Areas to be Measured

For microtiter plate analysis, the toolbox controls labeled Center and Outer specify the size of the inner circle and outer torus scanned to measure each well. The number in the center of each of these controls indicates the diameter of the circles. Click on an arrow to increase or decrease the diameters. As the number is adjusted, the objects on the template grow larger or smaller, reflecting the changes.

When specifying the size of the area to be measured, exclude shadows or other imaging artifacts. Depending upon the lighting conditions when the image was captured, there may be a crescent-shaped shadow obscuring part of the bottom of the wells, especially those near the edge of the microtiter plate. We recommend decreasing the size of the sampling area so that these regions are not included.

To measure the total density within each object, reduce the Center value to zero (0) and adjust the Outer value until the objects on the screen are slightly smaller than the areas of interest on the image. Reposition the corner objects using the mouse until the objects are properly aligned over the objects.

Measuring Density

Once the objects are sized and positioned correctly, click on the SCAN button in the tool box. The density values will be calculated and displayed under each object.

The values are the *average* pixel values within the object, adjusted to a scale of 0 to 100. (0 corresponding to black pixel values, and 100 corresponding to white pixel values.)

If both a center and an outer circle have been specified, two values are displayed. The upper value corresponds to the inner circle and the lower value corresponds to the torus.

The INVERT Box

The INVERT function reverses the relative density assignments so that 0 corresponds to white and 100 corresponds to black. If the image has dark areas of interest on a light background, then INVERT should be selected by placing an "X" in its box. (If the image has light areas of interest on a dark background, the INVERT option should not be activated.) Unlike the REVERSE button described in Chapter 3, this function does not alter the appearance of the image.

Removing Background using the Scan Blank Function

Since a microtiter plate is not completely transparent, it contributes to the density measured when samples are scanned. A non-uniform light source also affects the density measured. To ensure that the density measured is attributable only to the sample in the wells, scan a "blank" before scanning samples.

Load an image of a clean, unused microtiter plate and align the objects as described above. Once the array has been set up, click on the Scan Blank button. The average pixel gray value for each object (and torus) is measured and set to zero (0).

Now, when samples are scanned, the gray value of the blank is subtracted from that of the sample before it is scaled. This ensures that the density reported only reflects the sample in each well.

When Scan Blank has been selected, the background values obtained apply to all subsequent scans. To indicate that a background is being applied, the Scan Blank button changes to read Clear Blank. To turn off this background subtraction function, simply click on Clear Blank.

Common Export Result Feature

Once the analysis has completed then there is option to export the result. Either the results can print directly to a printer, or export the results as ASCII data for direct importation into Excel or other spreadsheet programs

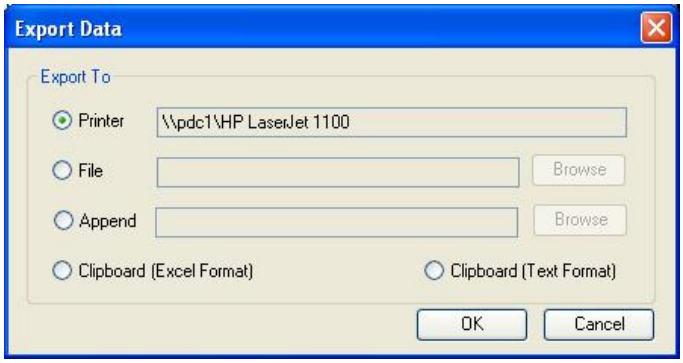


Figure 5.64 Common Export Dialog Box

Sending Data to a Printer

To send the data directly to the Video Printer or Default Printer, click on the Export button and click in the circle next to appropriate printer.

Sending Data to a File

If you would like to take the data from the system and import it into another computer, the data can be saved to a diskette or network which will allow you to open the data file on a separate workstation connected to the network.

Click on the Export button and then on the File option. Specify the path and file name to send the data file. The data is saved as an ASCII file and can be imported into most spreadsheet programs. ASCII is a very common file format output option for numerical data.

Appending Data to a Same File

Export dialog also allows you to append data to an existing exported file.
Note: This feature is applicable to AutoCount and Array analysis modules.

Sending Data to a Spreadsheet Program

To send the data results to Excel or other spreadsheet programs, click on the Export button, click on the clipboard option, then click on OK. If you have Excel or another spreadsheet program loaded on the system and running in the background, you can simply press the ALT and TAB keys simultaneously to move into the spreadsheet program. You can then import the data directly to the desired spreadsheet from clipboard.
Note: The spreadsheet program must be installed on the computer in order to export the data to that program.

When the appropriate export source has been selected, click on the OK button to send the data.

APPENDIX A: OPENING ALPHAVIEW™ FILES IN OTHER SOFTWARE PROGRAMS

Alpha Innotech-generated files have been tested in the software packages below. For successful imports, the command line is given.

Programs for the Macintosh® operating system were tested on a PowerMac® 8100/100AV. Results may vary for different software versions and/or hardware configurations.

		.TIF	.BMP	.GIF	.MAC	.PCX
Adobe Photoshop	**2.51 LE (Mac)**	Open	no	Open	Open As/TIFF	no
Adobe Photoshop	**3.0 (Mac)**	no	Open	Open		
Canvas	**3.5 (Mac)**	use ResEdit*	no	no	use ResEdit*	no
Microsoft Word	**6.0 (Mac)**	Insert Picture	Insert Picture	no	Insert Picture	no
Microsoft Word	**6.0a (Win)**	Insert Picture	Insert Picture	no	Insert Picture	Insert Picture
Microsoft Excel	**5.0 (Mac)**	no	no	no	no	no
Microsoft Excel	**5.0c (Win)**	Insert Picture	Insert Picture	no	Insert Picture	Insert Picture
NIH Image	**1.59 (Mac)**	Open	no	no	Open	no

*For instructions on using ResEdit™, see next page.

Additional packages, such as Claris Works and PowerPoint have also been tested. For these systems, it is necessary to save the file with a ".TIFF" extension in order for them to recognize the file as a TIF format.

If you have a specific software package that is not listed here, contact Alpha Innotech for a diskette. This PC-formatted disk contains a gel image saved as five of the file types that AlphaView™ can generate. Try opening each of these files in your software package to determine compatibility. We recommend that you start with the .TIF file, as this is the default Alpha Innotech file format.

Using ResEdit™

1) Save image in IS-1000/500 as a **TIFF** or **MACTIFF**

2) Obtain a copy of the freeware **ResEdit™** by downloading from Apple Computers through the Internet.

3) **Open ResEdit™.** An animated startup display will show up and continue until you click on the mouse or any key.

4) A dialog box will appear. **Open** the TIFF image. Another dialog box will appear asking if you want to **add a resource fork**, click on 'OK.'

5) Next go to the **File** pulldown menu and click on **Get Info for This File**

6) In the File Info window, change the Type to **TIFF** (instead of TEXT), and the Creator to **DAD2** (instead of DOSA). (Must be typed in all CAPS as shown here). Close the window and save changes. Quit ResEdit™.

7) After this procedure, the icon will change to a TIFF icon and the file may be opened **in Canvas.**

APPENDIX B: ALPHAVIEW™ MOLECULAR WEIGHT LIBRARY FILES

A library including the following DNA, RNA and protein molecular weight standards has been incorporated into AlphaView™. For information about using these standard files, see Section 5.2.

Values of size standards are given in basepairs.

AlphaQuant 1	AlphaQuant 2	AlphaQuant 3	AlphaQuant 4	AlphaQuant 5	AlphaQuant 6	AlphaQuant 7
10000	2000	5000	1000	500	2000	5000
8000	1800	4500	800	400	1000	3000
6000	1600	4000	700	300	500	2000
5000	1400	3500	600	250	250	1000
4000	1200	3000	500	200	100	500
3000	1000	2500	400	175		
2500	800	2000	300	150		
2000	700	1500	200	125		
1500	600	1000	100	100		
1000	500	700		75		
800	400	500		50		
600	300			25		
400	200					
200	100					
	50					

DNA Size Standards (in bp)								
HINDIII	PHIX174	BRL10BP	BRL50BP	BRL100BP	PRO100BP	BRL123BP	BRL1KB	BRLHIMW
23130	1353	200	500	1500	1500	4182	12216	48502
9416	1078	190	450	1400	1000	4059	11198	38416
6557	872	180	400	1300	900	3936	10180	33498
4361	603	170	350	1200	800	3813	9162	29942
2322	310	160	300	1100	700	3690	8144	24776
2027	281	150	250	1000	600	3567	7126	22621
564	271	140	200	900	500	3444	6108	19399
125	234	130	150	800	400	3321	5090	17057
	194	120	100	700	300	3198	4072	15004
	118	110	50	600	200	3075	3054	12220
	72	100		500	100	2952	2036	10086
		90		400		2829	1636	8612
		80		300		2706	1018	8271
		70		200		2583	517	
		60		100		2460	396	
		50				2337	344	
		40				2214	298	
		30				2091	220	
		20				1968	201	
		10				1845	154	
						1722	134	
						1599	75	
						1476		
						1353		
						1230		
						1107		
						984		
						861		
						738		
						615		
						492		
						369		
						246		
						123		

RNA Size Standards		Protein Markers (in kD)						
BRLRNA 1	BRLRNA 2	BRLPROT1	BRLPROT2	BRLPROT3	BRL10KD	NOVEXM12	SIGMAHMW	SIGMAPMW
1770	9490	87.0	200.0	43.0	200	200.0	205	190
1520	7460	60.0	97.4	29.0	120	116.3	116	108
1280	4400	53.0	68.0	18.4	110	97.4	97	89
780	2370	46.0	43.0	14.3	100	66.3	84	77
530	1350	40.0	29.0	6.2	90	55.4	66	61
400	240	34.0	18.4	3.0	80	36.5	55	41
280		29.0	14.3		70	31.0	45	36
155		27.0			60	21.5	36	
		21.0			50	14.4		
		15.0			40	6.0		
		6.5			30	3.5		
					20	2.5		
					10			

APPENDIX C: DATA TABLE DESCRIPTIONS

Single Channel Columns

Region Tab	
Band	Band Identifying Number
Sum	Sum of all pixel gray levels in Band
Percentage (%)	Percentage Sum =(Sum/ Total of all Band Sums)*100
Area	Area of Band Region in pixels
Average	Average pixel level of Band = (Sum/Area)
X Y Position	XY position of Band Region centroid
SD Pixel Levels	Standard Deviation of pixel gray levels in Band
Background Tab	
BC Sum	Background Corrected Sum = (BC Average *Area)
BC Average	Background Corrected Average = (Average – Bkgd Average)
Note	*Subtracting the Bkgd Average from the Average accounts for differences in area*
Bkgd Sum	Sum of all pixel gray levels in Background Region
Bkgd Area	Area of Background Region
Bkgd Average	Average pixel level of Background region = (Bkgd Sum /Bkgd area)
Bkgd XY	X Y position of Background region
Bkgd SD	Standard deviation of pixel gray levels in background region
Signal/Noise	Signal to Noise ratio of the band region = (Average– Bkgd Average) /Bkgd SD
Control Tab	
LCN Sum	Loading Control Normalized Sum = (LCN Average* Area)
LCN Average	Loading Control Normalized Average = (BC Average of Experimental Band/BC Average of corresponding Loading control) * mean of all loading controls.
Note	*If a background correction has not been applied, Average is used.*
LC Regions	Specifies the channel and region number used for each Experimental Band and corresponding Loading Control, (e.g. Red1/ Green 12)
PCN Sum	Positive Control Normalized Sum=(NCN Average *Area)
PCN Average	Positive Control Normalized Average = (LCN Average of experimental band / LCN Average of control band) *100.
Note	*If loading controls have not been applied, BC Average is used. If a background correction has not been applied, Average is used.*
PC Regions	Specifies the channel and region number used for each Experimental Band and corresponding Band Control, (e.g. Red1/ Red 6)
Fold Change	Fold Change of experimental band relative to control
Note	If LCN Average experimental band > LCN Average control band then fold change = LCN average experimental / LCN average control If LCN Average of the experimental band < LCN Average of control then fold change = (-1) * 1/(LCN average experimental / LCN average
Std Curve Tab	
"ng"	Values of Band region in Std curve in units as defined

Multi Channel Columns

Region Tab	
Band	Band Identifying Number
Blue Sum	Sum of all Blue channel pixel gray levels in Band
Blue Percentage	Percentage Blue Sum =(Blue Sum/ Total of all Blue Sums)*100
Blue Average	Average Blue channel pixel level (= Blue Sum/ Area)
Blue SD Pixel Levels	Standard Deviation of Blue Channel pixel gray levels in Band
Green Sum	Sum of all Green channel pixel gray levels in Band
Green Percentage	Percentage Green Sum =(Green Sum/ Total of all Green Sums)*100
Green Average	Average Green channel pixel level (= Green Sum/ Area)
Green SD Pixel Levels	Standard Deviation of Green Channel pixel gray levels in Band
Red Sum	Sum of all Red channel pixel gray levels in Band
Red Percentage	Percentage Red Sum =(Red Sum/ Total of all Red Sums)*100
Red Average	Average Red channel pixel level (= Red Sum/ Area)
Red SD Pixel Levels	Standard Deviation of Red Channel pixel gray levels in Band
Area	Area of Band Region in pixels
X Y Position	XY position of Band Region centroid
Blue/Green, etc	Ratio of indicated color values (Sum or BC Sum) for region
Background Tab	
Blue BC Sum	Background Corrected Sum = (Blue BC Average *Area)
Blue BC Average	Background Corrected Average = (Blue Average – Blue Bkgd Average)
Blue Bkgd Sum	Sum of all pixel gray levels in Blue Background Region
Blue Bkgd Average	Average pixel level of Blue Background region = (Blue Bkgd Sum /Bkgd area)
Blue Bkgd SD	Standard deviation of Blue pixel gray levels in background region
Blue Signal/Noise	Signal to Noise ratio of the Blue band = (Blue Average– Blue Bkgd Average) / Blue Bkgd SD
Blue ng	Results of Std. Curve quantitative analysis for blue channel data
Green ng	Results of Std. Curve quantitative analysis for green channel data
Red ng	Results of Std. Curve quantitative analysis for red channel data
Green BC Sum	Background Corrected Sum = (Green BC Average *Area)
Green BC Average	Background Corrected Average = (Green Average – Green Bkgd Average)
Green Bkgd Sum	Sum of all pixel gray levels in Green Background Region
Green Bkgd Average	Average pixel level of Green Background region = (Green Bkgd Sum /Bkgd area)
Green Bkgd SD	Standard deviation of Green pixel gray levels in background region
Green Signal/Noise	Signal to Noise ratio of the Green band = (Green Average– Green Bkgd Average) / Green Bkgd SD
Red BC Sum	Background Corrected Sum = (Red BC Average *Area)
Red BC Average	Background Corrected Average = (Red Average – Red Bkgd Average)
Red Bkgd Sum	Sum of all pixel gray levels in Red Background Region
Red Bkgd Average	Average pixel level of Red Background region = (Red Bkgd Sum /Bkgd area)
Red Bkgd SD	Standard deviation of Red pixel gray levels in background region
Red Signal/Noise	Signal to Noise ratio of the Red band = (Red Average– Red Bkgd Average) / Red Bkgd SD
Bkgd Area	Area of Background Region
Bkgd XY	X Y position of Background region
Control Tab	
LCN Sum	Loading Control Normalized Sum = (LCN Average* Area)
LCN Average	Loading Control Normalized Average = (BC Average of

	Experimental Band/BC Average of corresponding Loading control) * mean of all loading controls.
LC Regions	Specifies the channel and region number used for each Experimental Band and corresponding Loading Control, (e.g. Red1/ Green 12)
PCN Sum	Positive Control Normalized Sum=(NCN Average *Area)
PCN Average	Positive Control Normalized Average = (LCN Average of experimental band / LCN Average of control band) *100.
PC Regions	Specifies the channel and region number used for each Experimental Band and corresponding Band Control, (e.g. Red1/ Red 6)
Fold Change	Fold Change of experimental band relative to control
Note	If LCN Average experimental band > LCN Average control band then fold change = LCN average experimental / LCN average control If LCN Average of the experimental band < LCN Average of control then fold change = (-1) * 1/(LCN average experimental / LCN average

APPENDIX D: BIAS AND DARKMASTER UTILITY

The FluorChem Q is shipped with a set of darkmaster and bias files that meet the requirements of most commonly encountered imaging applications (1, 4, and 8 mins). Some applications, however, such as extremely low level signal detection with lengthy exposure times may benefit from generating specific darkmaster files matched to the exposure time of the application for optimum performance. AlphaView™ software includes a utility for generating bias and darkmaster files. Note that a darkmaster for given time need only be constructed once. Subsequent imaging applications will then apply the optimum darkmaster from the set of all darkmasters in the system.

CCD images exhibit a non-zero black level (bias) and temperature/time dependant levels (primarily thermally generated electrons called dark current) which are typically quite stable and can be corrected. Once there levels are characterized they can be removed from the image under consideration. This process is uniformly referred to as image calibration (along with flat field correction) to produce an image that contains only signals from the object under test and some residual noise from the original error sources.

The minimum requirements for accurate determination of bias and dark current levels is thermal stability and light tightness. Any fluctuations in temperature will alter the bias and dark current levels. Any light leaks will result in an additional source for errors that will not be correctly removed from images under consideration.

Bias and darkmaster files are created by collecting a set of 16 images that are combined in such a way to reduce the noise levels and reject spurious signals (such as cosmic rays, etc). A set of such files for darkmasters are created at different exposure times to create a library of darkmaster files. The files are logged into the program folder and used for all subsequent imaging sessions.

When an object is imaged the exposure time of the image is used to select the darkmaster with a similar exposure time and the darkmaster is scaled such that the dark current levels match that of the image under consideration. The bias and scaled darkmaster files are then subtracted from the image under consideration which results in an image with no bias (offset or black level errors) and elimination of dark current signals. As with any calibration method there are still noise artifacts that remain in the image after correction. At the time of acquisition an image contains the actual bias level plus bias level noise (also called read noise) in addition there is the dark current signal and its noise signals. The bias and darkmaster calibrations remove the bias and dark current signals but the noise from those signal remains (other techniques must be employed to address the residual noise-like frame averaging).

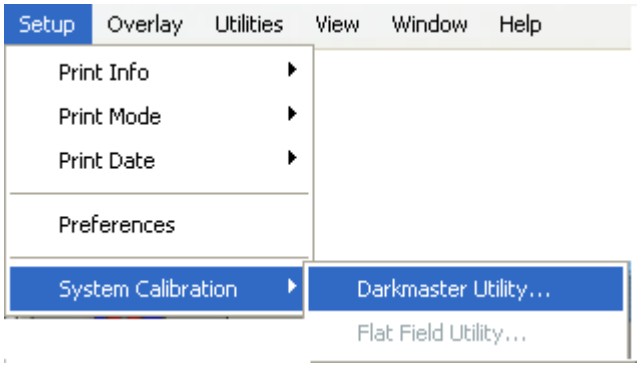
The best darkmaster images will have a similar exposure time to that which is typically used. The utility allows building darkmaster files of any exposure time (in 1 minute increments) so a darkmaster with a time that matches the typical exposure time can be constructed. It is best to have the darkmaster exposure time be greater than the time of interest. For example if 4.5 minutes is a typical time then a 5 minute darkmaster would be best. Shorter or much longer times will still work but optimal dark current correction occurs when using darkmaster exposure times greater than the test image exposure time (but no too much longer-round up to the next highest minute).

A procedure to use the utility to construct bias and darkmaster files is listed below.

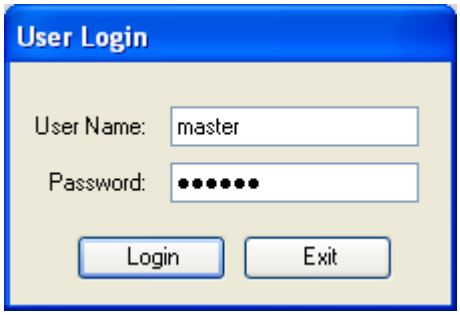
1. Allow camera to reach temperature and stabilize (at least 30 minutes after power on is recommended.

2. Put a cap on the lens mounting plate to block all light from the camera head. A lens cap will leak light. Creating darkmaster file with the camera and lens mounted to a cabinet is not recommended. If the camera cannot be dismounted then close the aperture and position the most opaque filter under the lens and turn off the cabinet before proceeding.

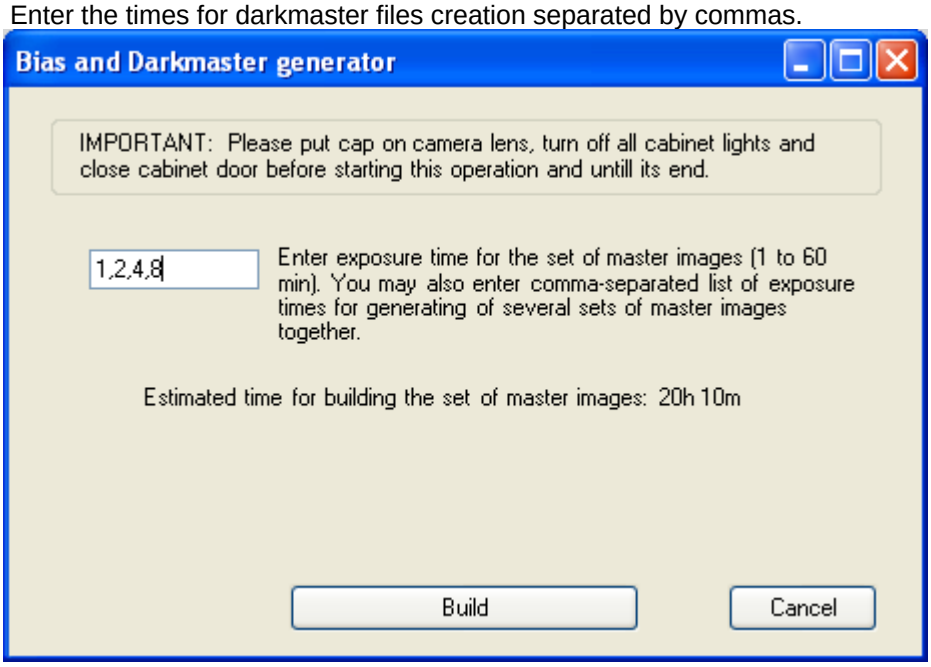
3. Access the utility from the application menus



4. Login to the utility (call tech support for the password)



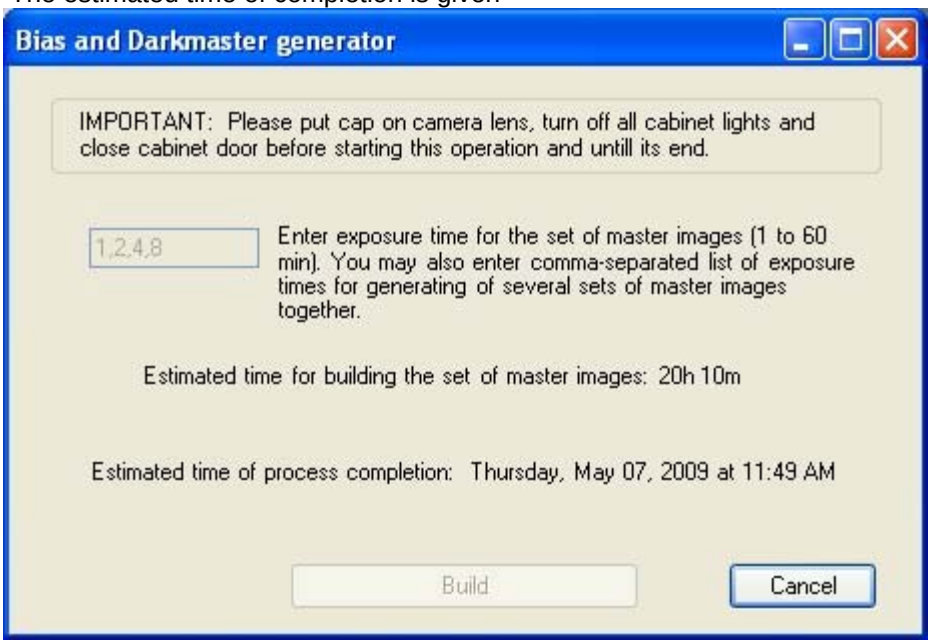
5. Enter the times for darkmaster files creation separated by commas.



6. If bias files do not exist they will be created automatically. If only bias files are desired then enter a time of 0 minutes.

7. Notice that the times to create the calibration images are quite long so please plan accordingly.

8. The estimated time of completion is given



9. A final notice is given when the process is complete.

APPENDIX E: FLAT FIELD CALIBRATION

The flat field calibration procedure is performed at installation or at the factory. Flat field files are provided for the most commonly encountered imaging situations. The purpose of the flat field calibration is to remove significant illumination and detection path non-uniformities from acquired images. Properly calibrated fluorescent images will be uniform for accurate quantification of signals levels within 5% at any position in the image.

A flat field calibration is only valid for images acquired under identical imaging conditions specific to that flat field file (not including exposure time or binning factor). A specific flat field file calibrates both the illumination intensity field and the image formation optics (lens and filter) and sample position within the cabinet. Consequently any change to either the illumination field or the imaging optics will degrade the calibration. Examples of such changes are positioning the sample at a different level within the cabinet, changing lens focus and aperture settings, replacing filters, or repositioning the camera and lens bracket.

The flat field calibration is applied automatically as part of the image acquisition process when a matching flat field is present in the application directory (C:\Program Files\FluorChem Q). If a matching flat field file is not present, then a flat field calibration is not applied. Also the flat field calibration may be disabled in the camera setup and preview window during acquisition.

The flat field image naming convention follows the format “FlatNxM.tif” where N is an index that lists the excitation light source and M is an index that lists the emission filter wheel position

Flat Field Calibration

The flat field calibration procedure is performed at installation or at the factory. Flat field files are provided for the most commonly encountered imaging situations. Flat field calibration corrects illumination and detection path non-uniformities from acquired images. Properly calibrated fluorescent images should have less than 5% non-uniformity across the field of view (12cmx12cm). Flat field calibration is appropriately utilized when no changes are made between flat field calibration and the imaging of biological samples. A specific flat field file calibrates both the illumination intensity field and the image formation optics (lens and filter) and sample position within the cabinet. Consequently any change to either the illumination field or the imaging optics will degrade the calibration. Examples of such changes are positioning the sample at a different level within the cabinet, changing lens focus and aperture settings, replacing filters, or repositioning the camera and lens bracket.
The flat field calibration is applied automatically as part of the image acquisition process when a matching flat field is present in the application directory (C:\Program Files\AlphaView Q). If a matching flat field file is not present, no flat field calibration is performed. Also the flat field calibration may be disabled in the camera setup and preview window during acquisition.

Flat Field Calibration Procedure:
The appropriately named flat field file will be placed in the application directory and subsequently applied to image acquisition at those illuminations and filter settings. The

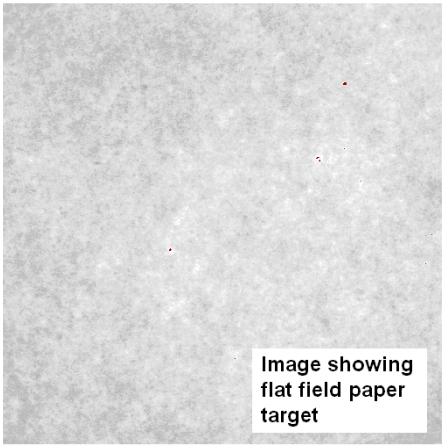
flat utility applies a smoothing function to the acquired image to remove irregularities due to mottling or fibers in the paper.

STEPS:

1. Place a uniform target such a piece of white laser printer paper at the sample location that will be used for fluorescent imaging.

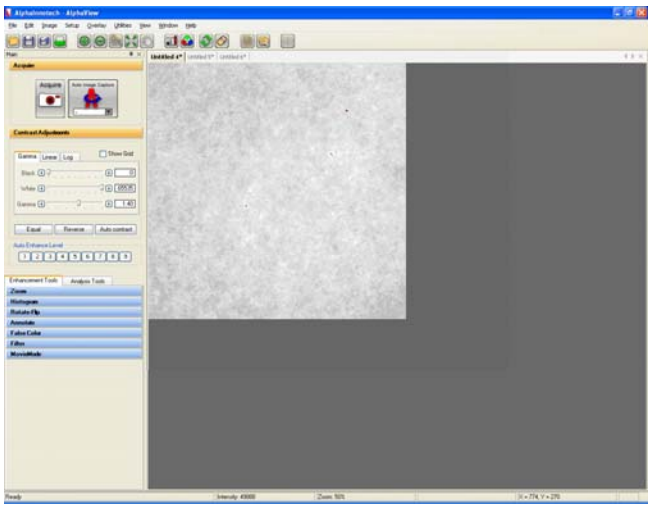
Flat field target inside FluorChem Q

2. Focus on the surface of the flat target (paper target shown below). Note: It is important to set lens focus and aperture settings to those to be used for image acquisition.



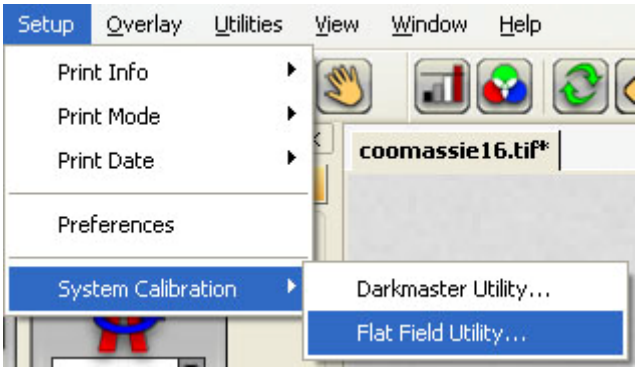
Example flat field target image

3. Acquire a grayscale image of the target with the desired illumination and filter settings. Use the top speed resolution setting possible (Normal/Ultra) and Zoom ROI setting = 1. The image should be generally bright with pixel gray levels between 50% and 90% of saturation (intensity values = 30,000 - 58,000). A few saturated (red) pixels are allowed.



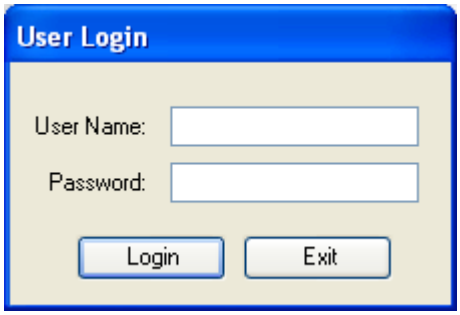
Example AlphaView ™ screenshot

4. Select **Flat Field Utility** from Setup Menu



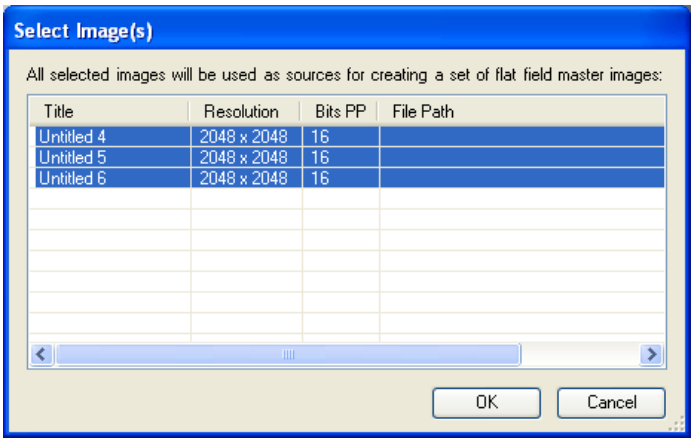
Flat field menu path

5. Complete login screen security entry. Default is provided below:
   User name: master
   Password: master



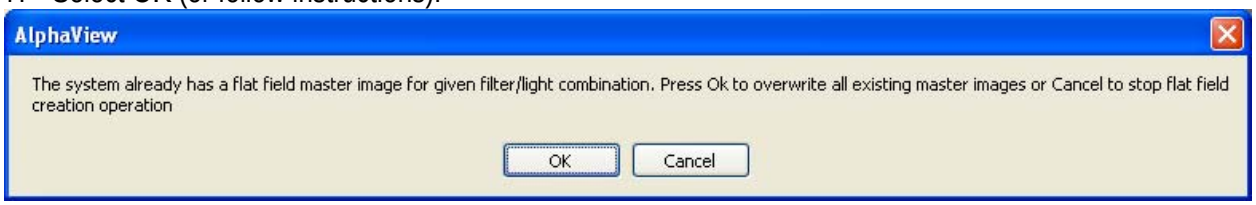
Security Login Screen

6. Image selection screen – review images created for flat field calibration. Images shows should match images acquired for flat field calibration.



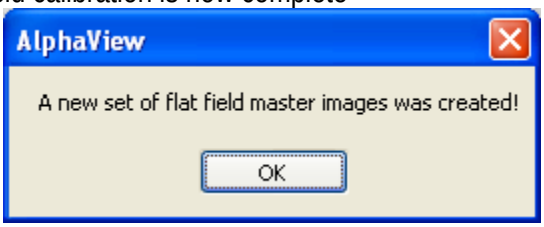
Flat field file imager selection

7. Select OK (or follow instructions).



Info screen

8. Select OK. Flat field calibration is now complete



Info screen

APPENDIX F: DATA INTERPRETATION

Overview

In a typical Western blot experiment the expression levels of one or more experimental proteins are analyzed to determine the effects of various treatments or conditions. The appropriate controls must be included on westerns blots as a number of factors can influence the proper interpretation of the signal levels extracted from multicolor images especially when comparing signal levels from different color channels.

A loading control is a protein that is unaffected by the treatments and whose signal level can be used to normalize the signal levels of the experimental proteins for differences in the amounts of sample loaded in each lane. An untreated sample should also be included to provide a negative (untreated) control for each experimental protein. Alternatively a positive or reference control sample may be included to provide a control.

Consider the case in which the loading control protein is labeled with Cy2 (Blue channel), and two experimental proteins are labeled with Cy3 (Green channel) and Cy5 (Red channel) respectively. An untreated sample is included with a number of treated samples. The objective is to determine the effects of the treatments on the expression levels of experimental proteins and to determine the relative responses of one experimental protein to the other. It is assumed that replicates will be analyzed to provide significance.

First create regions for the experimental bands and loading controls and subtract background as described above. Then perform the loading control normalization to adjust the signal levels for the Cy3 bands and Cy5 bands for differences in the amount of sample loaded per lane. Note that the loading control is in the Blue channel and the experimental proteins are in the Green and Red channels respectively. The reason that the loading controls and experimental proteins may be in different color channels is that the relative difference between channels is factored out during the loading control normalization calculation.

For Green channel signals (g1, g2 and g3) and Red channel signals (r1, r2 and r3) and Blue channels signals (b1, b2 and b3) where the values correspond to BC Sums, the results after loading control normalization are:

(g1/b1)*bm, (g2/b2)*bm, (g3/b3)*bm
(r1/b1)*bm, (r2/b2)*bm, (r3/b3)*bm

Bm: mean of the Blue loading controls.

Now consider that the Blue signal level doubles relative to the Red and Green signal levels, now the ratios are:

(g1/**2**b1)*__2__bm, (g2/**2**b2)*__2__bm , (g3/**2**b3)__2__*bm
(r1/**2**b1)*__2__bm, (r2/**2**b2)*__2__bm, (r3/**2**b3)*__2__bm

The twos (**2**) factor out, so the relative change in Blue to Green and Red signal levels does not effect the loading control normalization. (Note that even without multiplying by the mean of the loading controls the relative values of the Red/Green bands would still factor out.)

Next perform band control normalization for both the Cy3 Green bands and Cy5 Red bands.

Use the Cy3 Green negative control to normalize the Cy3 experimental bands and use the Cy5 Red negative control to normalize the Cy5 bands. (Note that band normalization uses the loading control normalized values.)

The band control normalized data provides the relative change in expression level of each Cy3 labeled experimental protein relative to the its untreated control and the relative change in expression level of each Cy5 labeled experimental protein relative to its untreated control. The relative changes of the Cy3 protein to the Cy5 labeled protein is then determined by comparing the changes of each to its respective control.

For example consider that the Cy3 band in lane 3 is decreased by 2 fold relative to its untreated control and the Cy5 band in lane 3 is increased by 3 fold relative to its untreated control. This yields a relative change of the Cy3 band in lane 3 the to the Cy 5 band in lane 3 as a decrease of 6 fold.

Note that comparing the Red signal levels to the Green signal levels directly is only valid for the conditions of that single blot. Replicate blots, or even a second image of the same blot, may have different relative Red and Green signals levels due to different labeling conditions, reagent concentrations, exposures times, or photo bleaching effects.

When analyzing replicate multicolor blots it is good practice to acquire images at exactly the same acquisition settings. In cases where this may lead to less than optimum images for subsequent replicates due to changes in sample loading, labeling or other experimental factors, follow the data analysis procedure described above to compare the data from replicates.

Note: In the Data table there are columns available for Blue/Green, Blue/Red, etc. These columns display the ratio of the indicated channel Sums (or BC Sums) for that region and are intended for analysis of dual labeled bands or spots as may encountered in dot blots or macroarrays. These raw ratios are somewhat arbitrary as they depend on the relative exposure times of the channels but do provide the relative values of the channel signal levels for that region. Do not confuse these ratios with the LC regions or PC regions which indicate which regions are selected as control and experimental bands respectively.

APPENDIX G: FULLY WORKED EXAMPLES OF MULTICOLOR BAND ANALYSIS

Example 1: Phosphorylation

The samples are a time course study that monitors the up/down regulation of phosphorylation following treatment. Red and blue channel signals are detected as overlapping bands in this example. The red channel represents signals from an antibody that only binds the phospho-specific forms of a protein (pX=background corrected average from the red channel data). Blue channel data represents the signal from an antibody that binds all isoforms (phosphorylated and nonphosphorylated) of the protein (X+pX=background corrected blue channel data) and is referred to as the total protein signal.

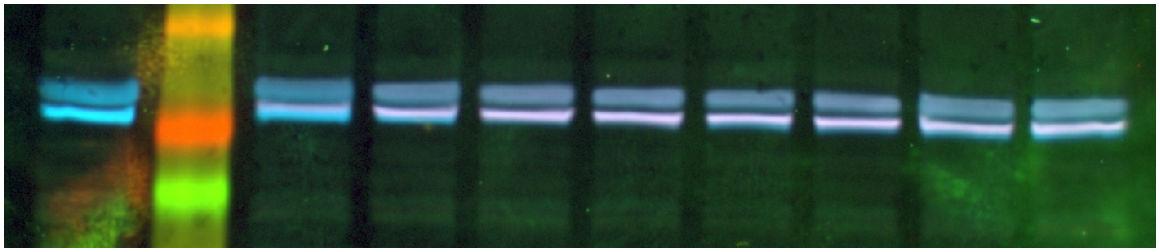
In this case the total protein signal can be used as a loading control. The blue channel data represents total protein (X+pX), while the red channel data represents only phospho-specific protein. So, by designating the blue channel data as a loading control and the red channel data as the experimental sample, an LCN for the phospho-specific protein is calculated as LCN=pX/$(X+pX)_{Lane\ N}$.

Lane 1 contains the untreated reference sample and the ratio of pX/(X+pX) in this lane is used as a baseline reference (Baseline= pX/$(X+pX)_{Lane\ 1}$). So, the ratio of phospho-protein to total protein in the untreated reference sample represents the Baseline.. All other lanes (except the lane with markers) are used to calculate the ratio pX/$(X+pX)_{Lane\ N}$ relative to the Baseline.

The band control normalization tool is used to determine Fold Change. The red channel of lane 1 is designated as the band control, while the red channel of the other lanes is designated for experimental bands. Since the red channel of these bands have already been subjected to LCN normalization, this provides a Fold Change determination with the following relationship:
Fold Change = pX/$(X+pX)_{Lane\ N}$ / pX/$(X+pX)_{Lane\ 1}$

The Fold Change in phospho-specific protein is calculated relative to the Baseline expression levels.

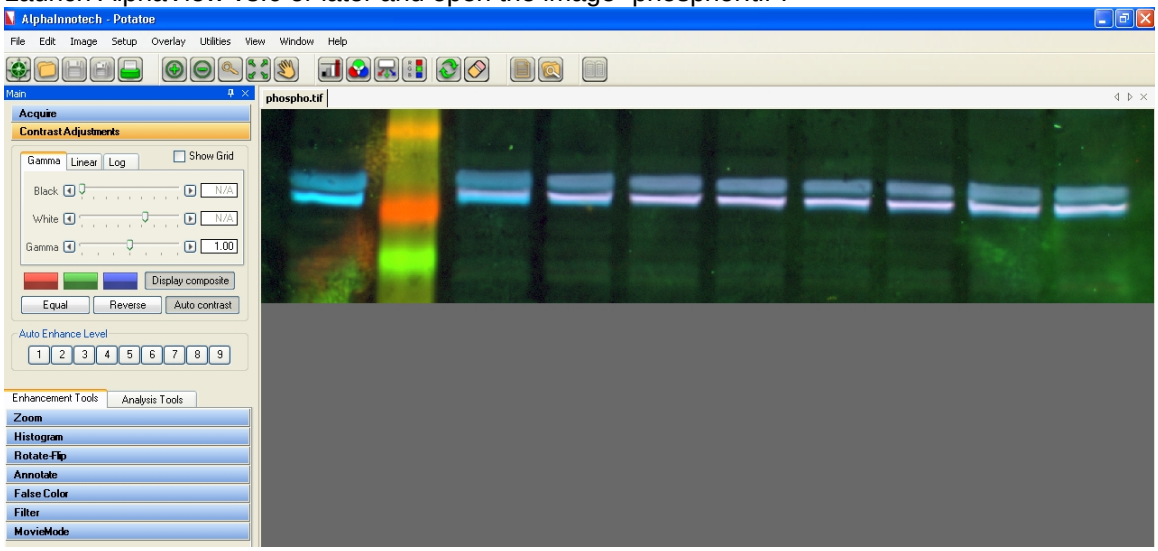
The image below shows a portion of the image that contains the primary bands.



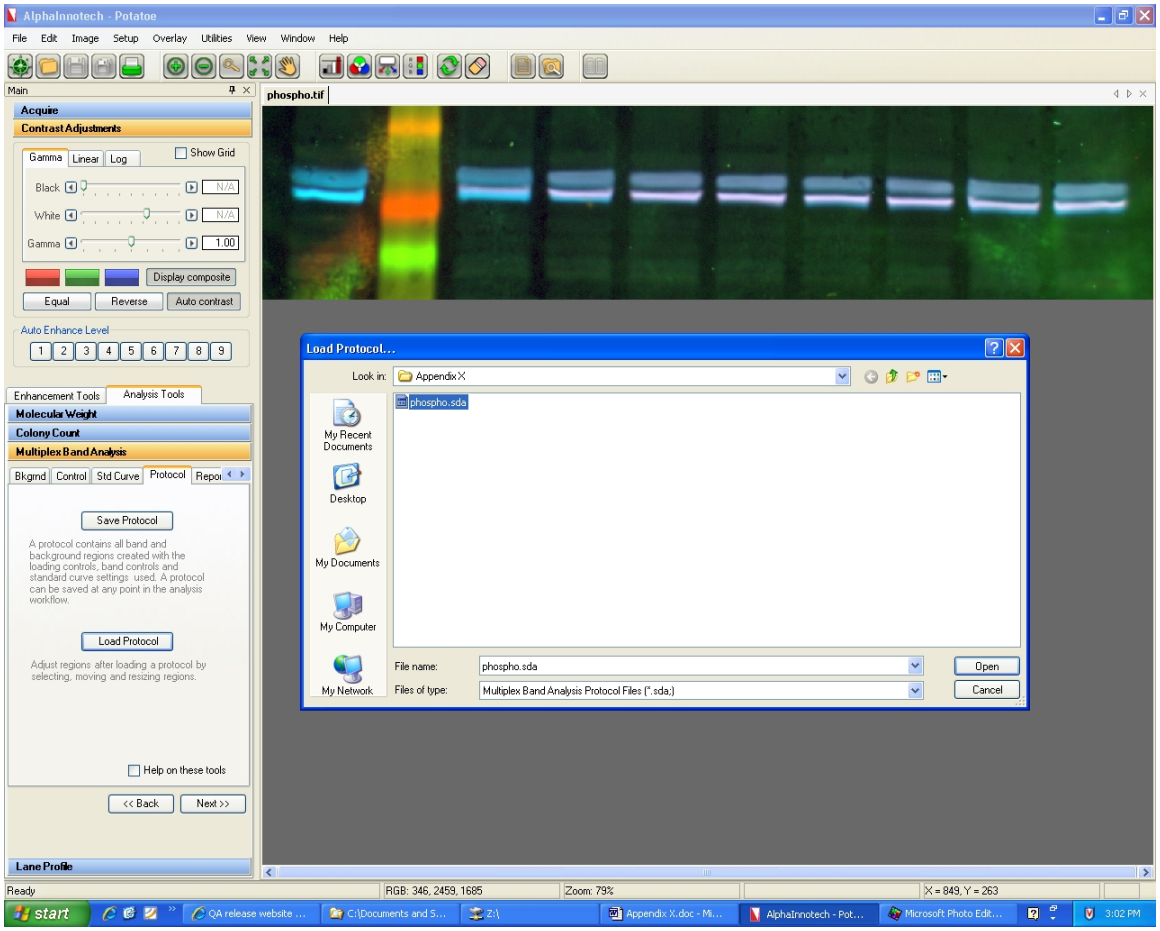
Section of blot showing the primary bands of interest

In above Figure, lane 1 contains the untreated reference sample. The ratio of pX (red channel data) to X+pX (blue channel data) serves as the Baseline level of phosphorylation. In this case phosphorylation does not greatly impact molecular weight so phospho- and non-phospho bands overlap on the blot.

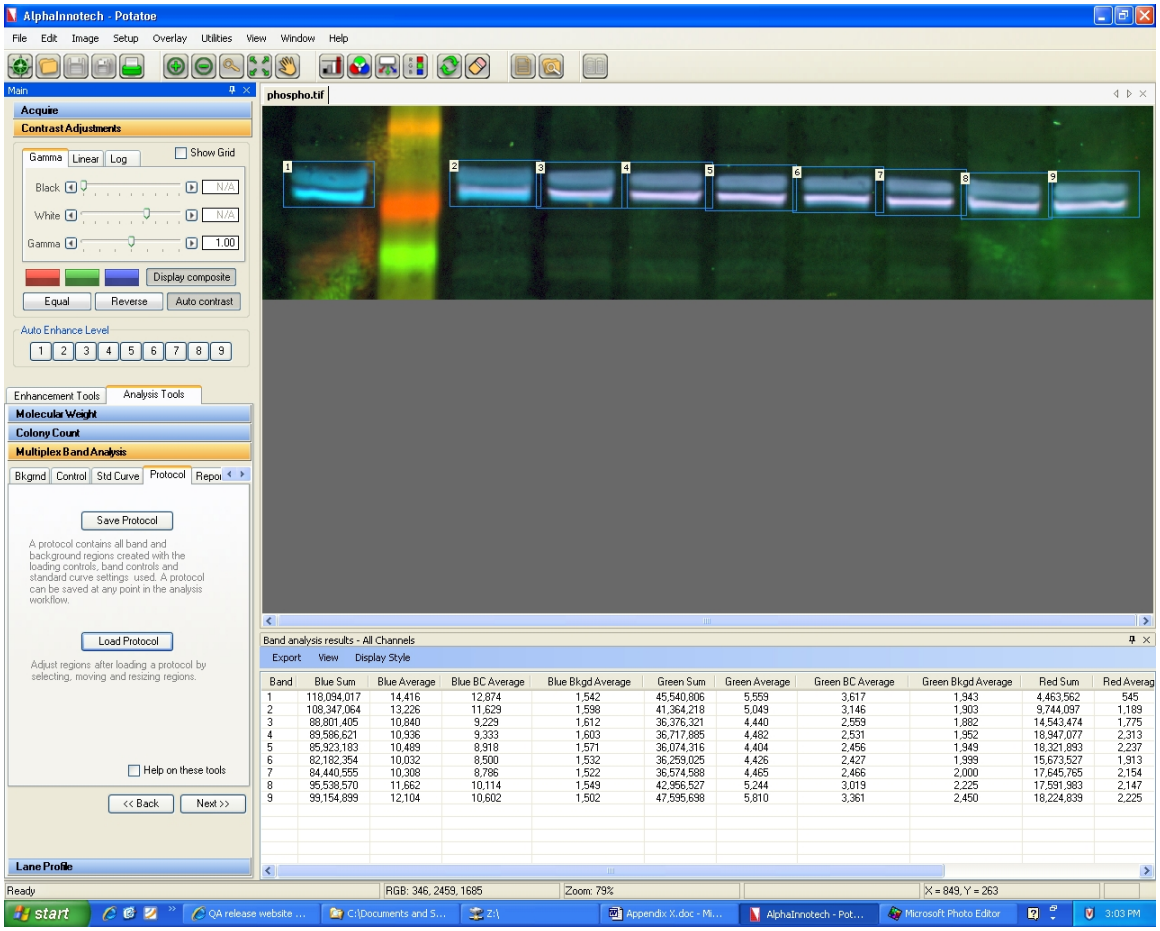
Launch AlphaView v3.0 or later and open the image “phosphor.tif”.



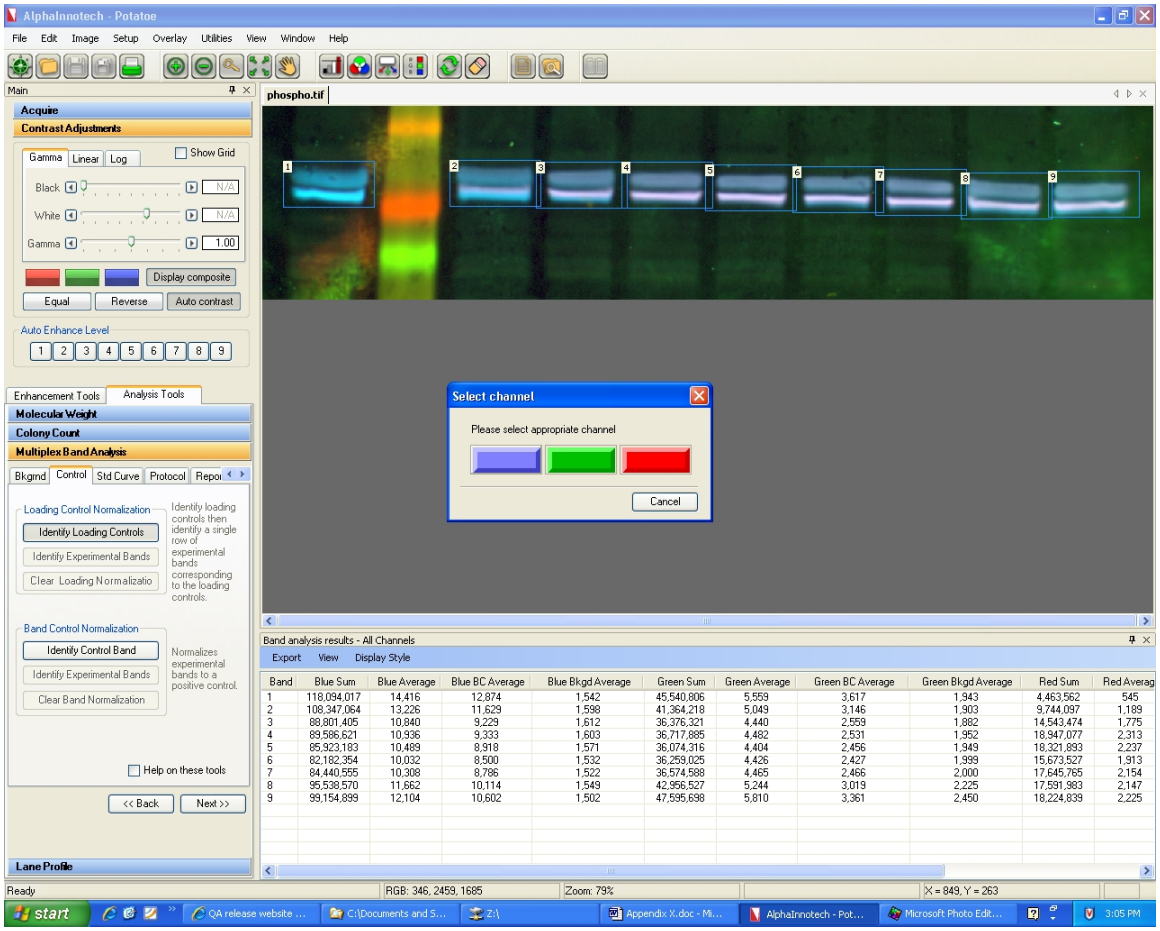
Open the Analysis tab. Select Multiplex Band Analysis. Select Protocol tab and Load Protocol. Browse to “phospho1.sda” protocol.



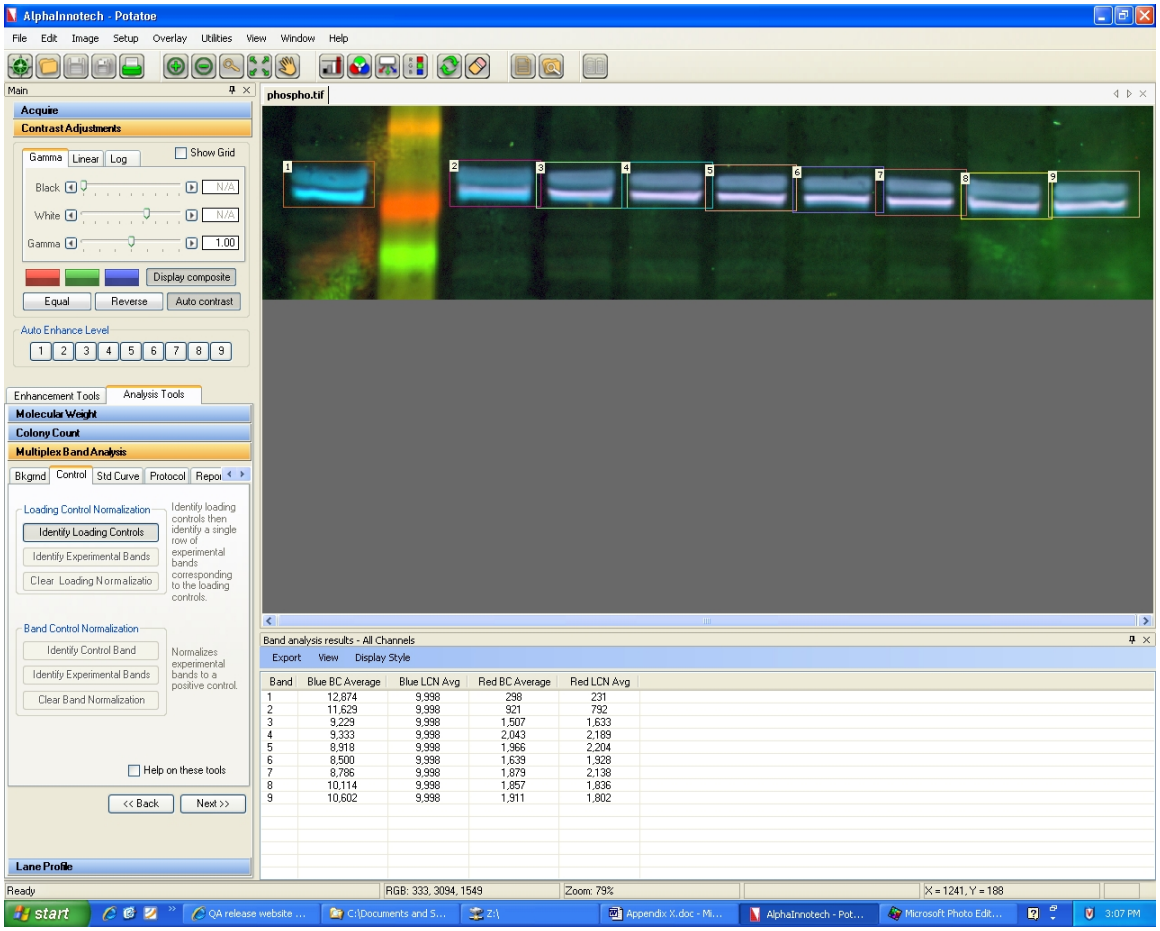
This saved protocol has Regions and a Local Background Correction defined. In this example the Regions have been purposefully defined larger than the bands and contain many pixels of background signal.



Select the Control Tab and select Identify Loading Controls. In this example Loading Controls are represented by the blue channel.



Select the Blue channel and then select all Regions to identify them as Loading Controls.



Click the Identify Loading Controls button to de-activate the selection tool. Notice the data columns in the data table show the blue and red values for the background-corrected average (BC Average); Local Background correction was pre-defined for this protocol.

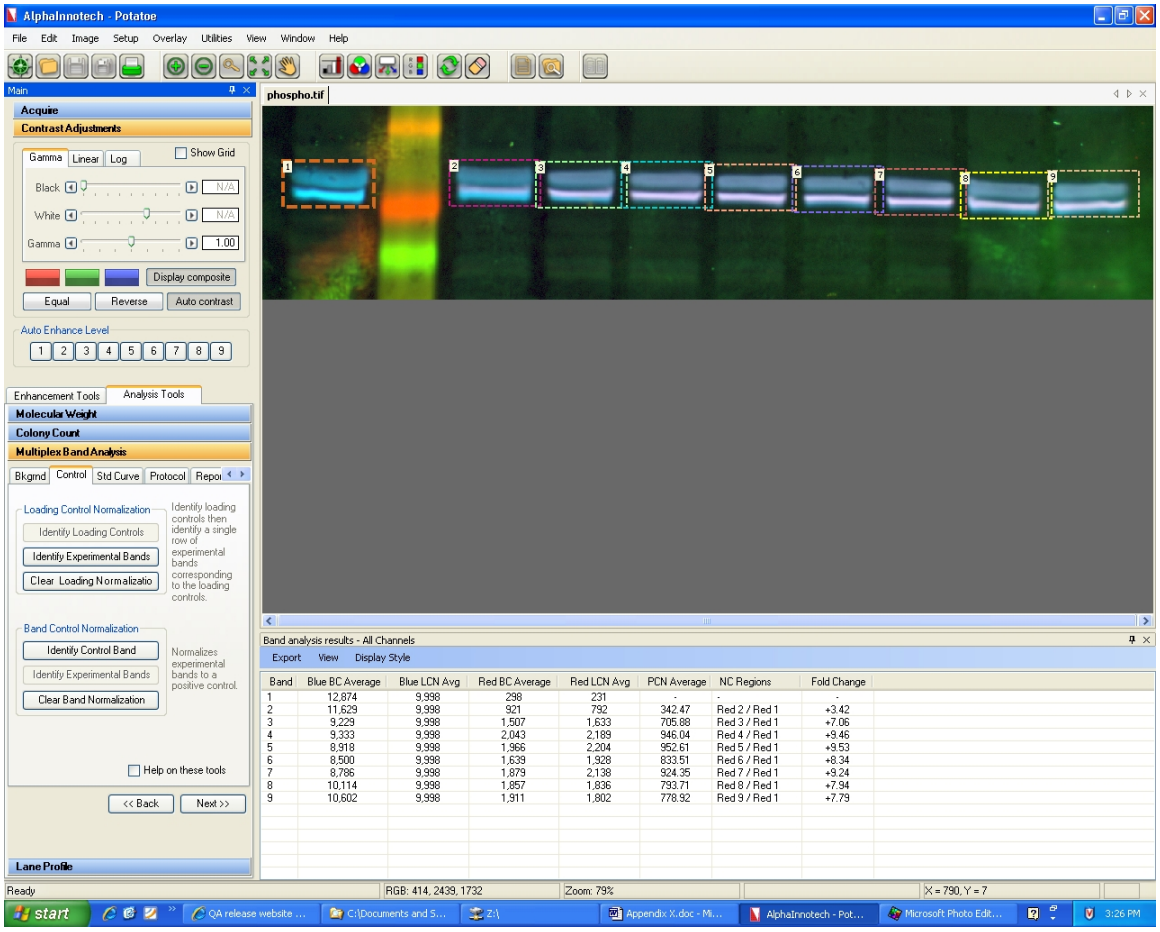
The data table also displays the Loading Control Normalized data (LCN Avg) Note that all values for Blue LCN Avg are identical. This is expected, because the Blue channel is designated as the Loading Control, and the calculation for the Loading Control determines the mean intensity for all Loading Controls. In this example the Blue Loading Control values are calculated from the Blue BC Avg, which represents total protein (X+pX) for each lane. Ideally identical quantities of total protein are loaded in each lane, but in reality slight variations in loading usually occur from lane to lane.

Note that the Red LCN Avg is calculated: Red BC Avg * Blue LCN Avg/Blue BC Average, using the respective values for the band of interest (e.g. for band #1: Red LCN Avg = 298*9998/12874=231.

The Red LCN Avg values now represent the pX/(X+pX) ratio for each corresponding band.

In this example it is not necessary to designate separate Experimental Bands. The defined Control bands function as experimental bands without explicitly being defined as such.

Select Identify Control Band from the Band Control Normalization section and select the red channel of region #1 (primary band of lane 1), which represents the untreated sample.



Select Identify Experimental Bands and select regions 2-9. De-activate this tool by pressing Identify Experimental Bands or right-clicking. Note the data column Fold Change is populated. The Fold Change column represents up/down regulation in phosphorylated protein relative to baseline levels of phosphorylation in the reference sample (Lane 1).

The Fold Change value is calculated using the Red LCN Avg values for each band compared to the Red LCN Avg value for the control band (band #1) (e.g. for band #2: Fold Change = Red LCN $Avg_{Band\ \#2}$ / Red LCN $Avg_{Control\ Band\ \#1}$ = 792/231=3.42).

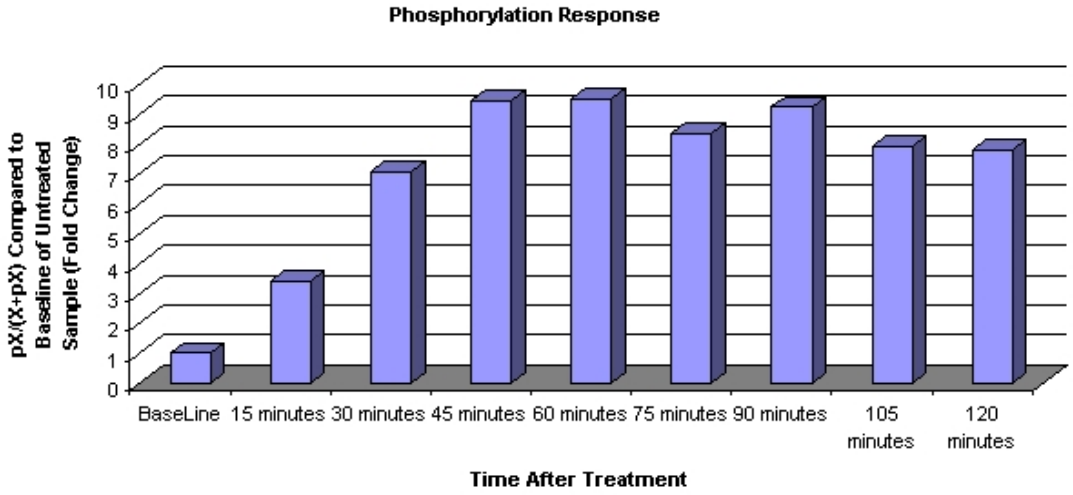
Recall that Red LCN Avg = Red BC Avg *Blue LCN Avg/Blue BC Average for each band, and recall that Red BC Avg = pX, Blue BC Avg=(X+pX).

When the value for Fold Change is calculated: Red LCN $Avg_{Band\ \#2}$/Red LCN $Avg_{Band\ \#1}$ the following substitution can be made:
Fold Change = $pX_{Band\ \#2}$*Blue LCN $Avg/(X+pX)_{Band\#2}$ / $pX_{Band\ \#1}$*Blue LCN $Avg/(X+pX)_{Band\#1}$ and notice the value Blue LCN Avg cancels, leaving the desired result.

Fold Change$_{Band\#2}$ = $\frac{pX_{Band\#2}/(X+pX)_{Band\#2}}{pX_{Band\#1}/(X+pX)_{Band\#1}}$, which is the level of phosphorylation relative to the total amount of protein for band #2 compared to the baseline level of phosphorylation relative to the total amount of protein for band #1 (the untreated sample).

Repeat the above for the other bands to complete the analysis.

The data table can be exported and Microsoft EXCEL utilized to prepare graphs.

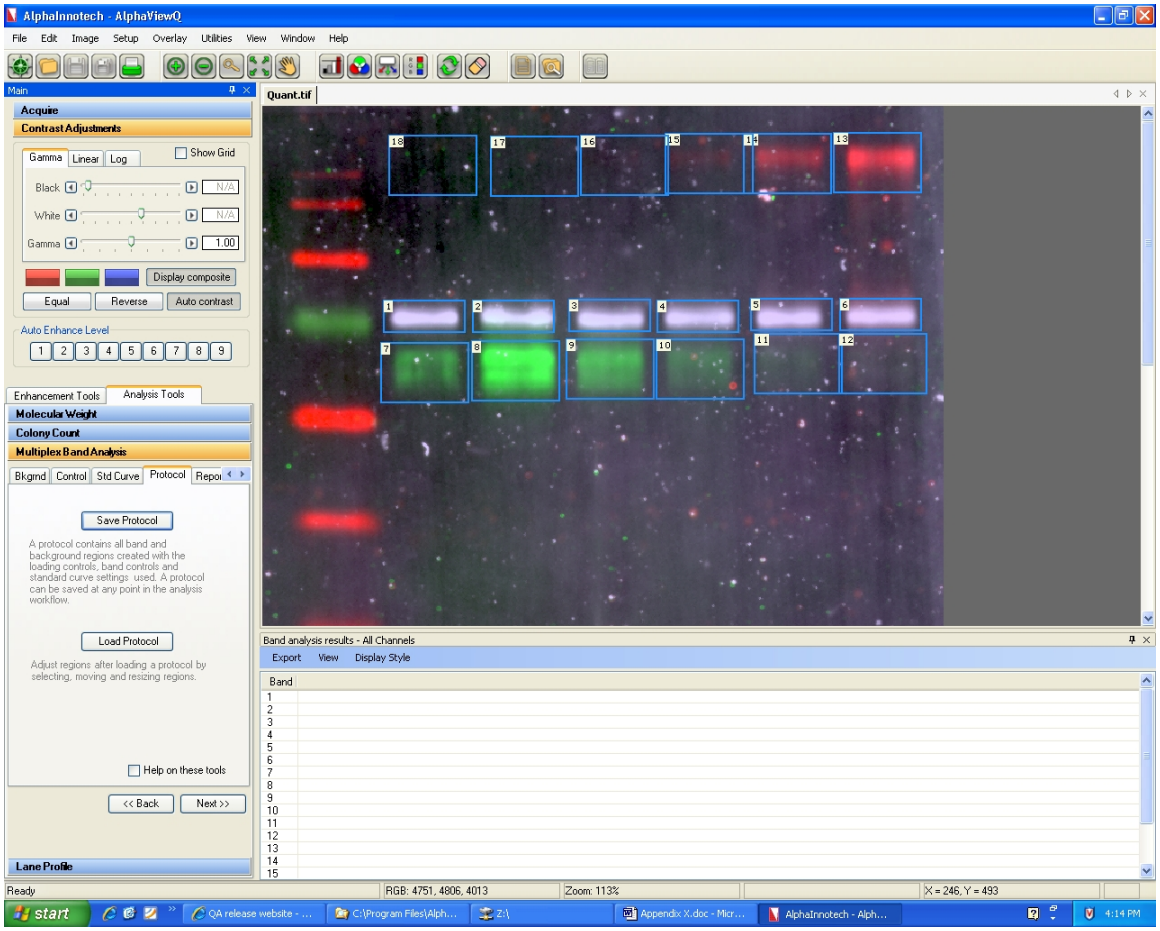


Example #2 Quantitation across color channels

In this example the multicolor blot includes a dilution series in the red channel and a treatment course indicating expression level changes for a phosphorylated isoform in the green channel. There is also a specially constructed control sample that serves as a loading control, as well as a control for color channel normalization (Color Control). In this example, the goal is to determine the absolute concentration of the phosphorylated isoform (green signal) using the dilution series in the red channel as a standard curve.

The Color Control is constructed using equi-molar concentrations of a reference protein that has been separately labeled with the GE DIGE minimal dye labeling kit using CY2, CY3 and CY5 dyes. Therefore, the Color Control has a known quantity of material in all three color channels (in this case the relative ratios are 1:1:1). This Color Control permits direct comparison of the red, green and blue channel data when the corresponding signals are normalized to the Color Control signals; this process is termed Color Control Normalization or CCN.

Following the steps in the above Example #1, launch AlphaView and open the image “Quant.tif”. Then, open the Multiplex Band Analysis module and load the protocol “quant.sda”.



In the figure above, bands 1-6 are the Color Control bands serving as loading controls. Any color channel can be used to perform the Loading Control Normalization.

Bands 7-12 of green channel data represent the signal from the phospho-specific antibody, with band 7 being the reference sample not subject to treatment.

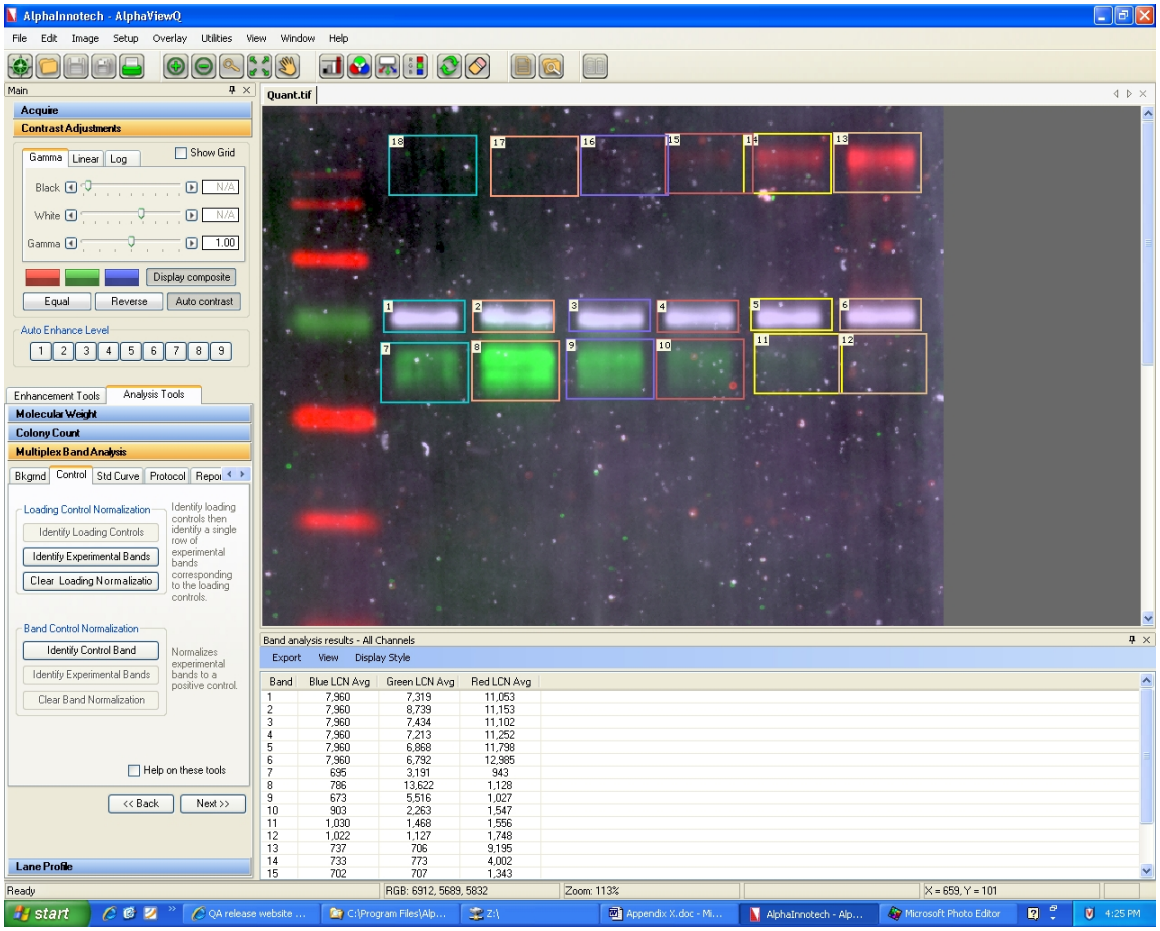
Bands 13-18 of red channel data represent the dilution series with the following amounts of protein from left to right 0, 0.4, 1.1, 3.3, 10, and 30 (nanograms).

Select the Background tab and select Local Background.

Select the Control Tab and Identify Loading Controls by choosing the blue channel and selecting bands 1-6. De-activate the Loading Control tool by selecting the Identify Loading Controls button (or right mouse click).

Select Identify Experimental Bands and select bands 7-12. The Identify Experimental Bands feature is automatically de-activated if the number of experimental bands matches the number of control bands.

Press the Identify Experimental Bands button again and select bands 13-18.



Notice the data table shows the Loading Control Normalized (LCN) values for all the bands and all the color channels.

Use the Band Control Normalization tool to determine the Fold Change in the phosphorylated samples relative to Band 7.

Select Identify Control Band in the Band Control Normalization section and select the green channel and Band 7.

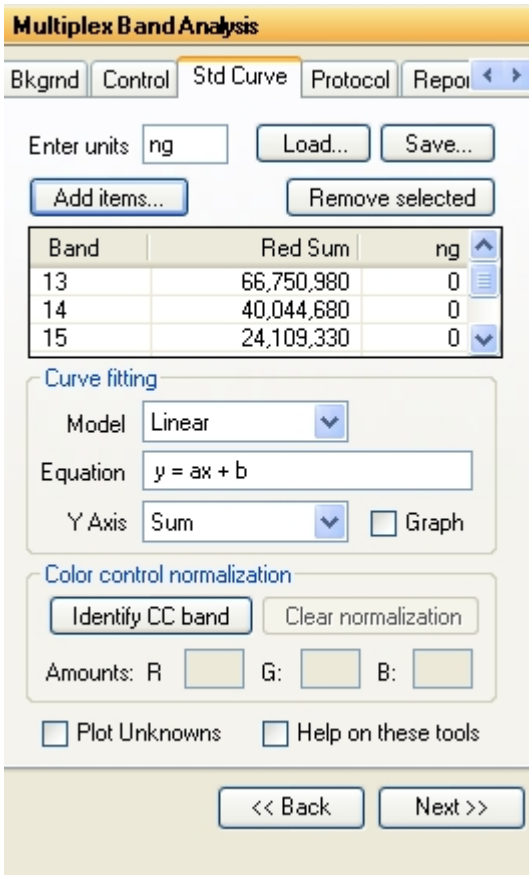
Select Identify Experiment Bands, and select bands 8-12. Fold Change values are populated in the data table.

Band analysis results - All Channels

Export View Display Style

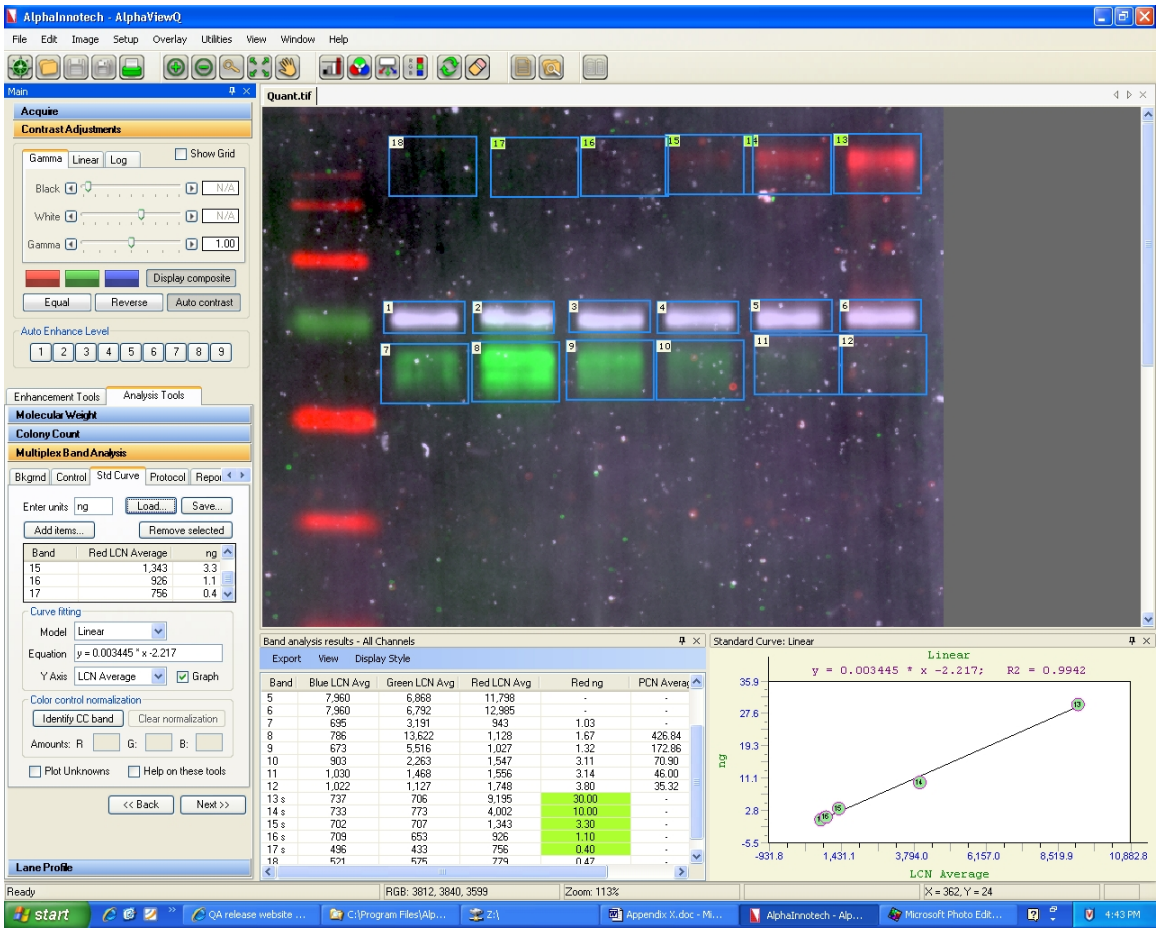
Band	Blue LCN Avg	Green LCN Avg	Red LCN Avg	PCN Average	Fold Change
3	7,960	7,434	11,102	-	-
4	7,960	7,213	11,252	-	-
5	7,960	6,868	11,798	-	-
6	7,960	6,792	12,985	-	-
7	695	3,191	943	-	-
8	786	13,622	1,128	426.84	+4.27
9	673	5,516	1,027	172.86	+1.73
10	903	2,263	1,547	70.90	-1.41
11	1,030	1,468	1,556	46.00	-2.17
12	1,022	1,127	1,748	35.32	-2.83
13	737	706	9,195	-	-
14	733	773	4,002	-	-
15	702	707	1,343	-	-
16	709	653	926	-	-
17	496	433	756	-	-

Select the Std Curve tab to construct a quantitative curve. Select Add Items..., and select the red channel. Select bands 13-18. Band 18 has a concentration of 0ng. De-activate the Add Items… tool by selecting Add Items… again.



In the Std Curve data table enter the known concentrations of protein for each standard. Select an appropriate Curve Fitting model and select an appropriate Y axis parameter

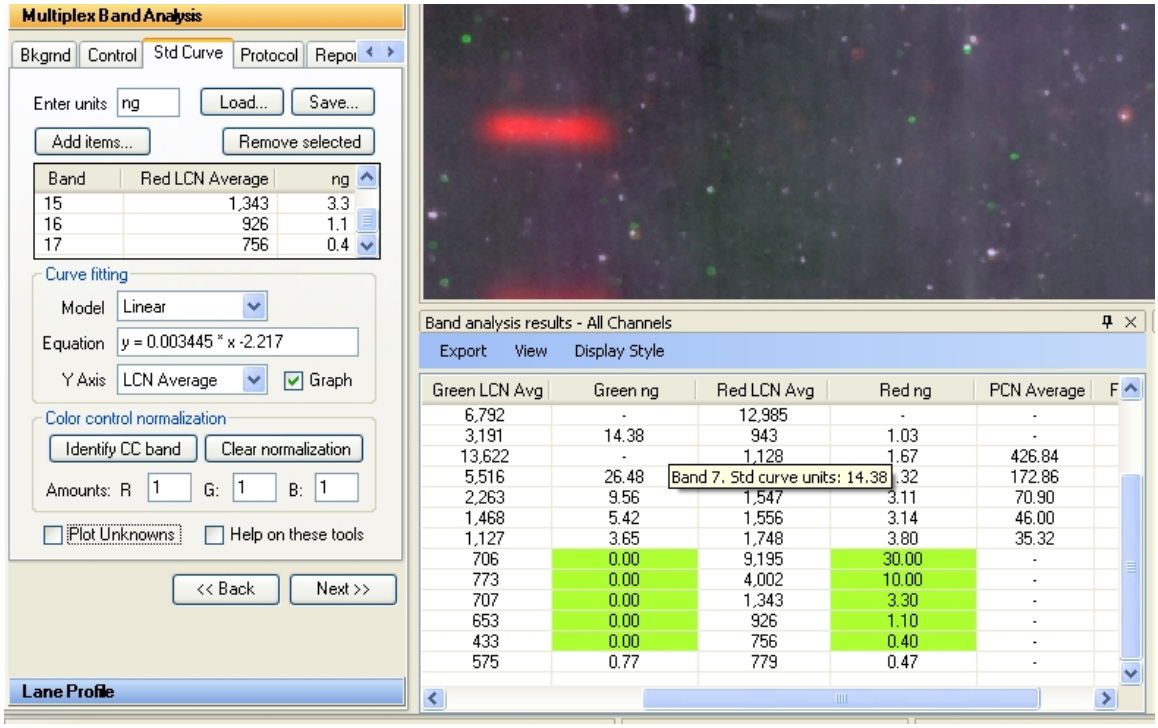
(LCN Avg is preferable in this example). Once the known concentrations are entered, the curve fit is calculated and a graph plotted.



In the figure above the known concentrations appear in the table of the Std Curve section, as well as in the results data table. A new column appears in the results data table displaying the calculated concentrations for the Experimental bands for the red channel. The standard concentrations are highlighted in green and the band number is marked with an “s” for Standard.

To determine the concentrations of phosphorylated protein (the green signal in bands 7-12), use the signals for the Color Control bands to make cross-channel calibrations of the mass standards.

Select Identify CC Band in the Color Control Normalization section and select band 1. In the Amounts section of Color Control Normalization, enter values for Red, Green and Blue. In this example an equi-molar ratio of 1:1:1 is represented.



In the figure above the values in the results data table display the nanogram concentrations of phosphorylated protein (represented by the green channel data) in the column labeled Green ng. Hover the mouse pointer over the column Green ng for Band 7, and the tool tip displays the band number and the data value. The value for Band 8 displays a "-" indicating the concentration is outside the range defined by the known concentrations used from the red channel data to construct the mass standard curve. Only values within the defined range are accurately quantified.

APPENDIX H: REGULATORY COMPLIANCE

FCC Statement:

This device complies with part 15 of the FCC Rules Operation is subject to the following two conditions

1) this device may not cause harmful interference, and
2) this device must accept ay interference received including interference that may cause undesired operation.

This Class [A] digital apparatus meets all requirements of the Canadian Interference-Causing Equipment Regulations.
Cet appareil numérique de la classe [A] respecte toutes les exigences du Réglement sur le matériel brouilleur du Canada.

Compliance/Safety: Electromagnetic Compliance (Emissions):
FCC (CFR 47, Part 15) Class A
IECS-003, Issue 3, Class A
VCCI V-3 / 2006.04
EN 61326: 1997/A1: 1998/A2: 2001/A3: 2003

Safety:
UL 61010 – 1: 2004 (2nd Edition); CAN/ CSA- C22.2 No 61010 – 1: 2004 (2nd Edition)
EN 61010 – 1: 2001 (2nd Edition)

Power Requirements

AC Input voltage rating: AC 100-120 V, 50/60 Hz, 3 A
AC 200-240V, 50/60 Hz, 3 A

Environmental Requirements (Example)

Operating temperature 0° to 40° C
Non-operating temperature -20° to 65° C
Operating altitude Sea level to 10,000 feet
Operating relative humidity 10% to 90%, non-condensing
Non-operating relative humidity 5% to 95%, non-condensing

• English

CAUTION — The power supply cord is used as the main disconnect device, ensure that the socketoutlet is located/installed near the equipment and is easily accessible.

• German

ACHTUNG — Zur sicheren Trennung des Gerätes vom Netz ist der Netzstecker zu ziehen. Vergewissern Sie sich, daß die Steckdose leicht zugänglich ist.

• French

ATTENTION — Le cordon d'alimentation est utilisé comme interrupteur général. La prise decourant doit être située ou installée à proximité du materiél et être facile d'accés.

English

Warning

This product relies on the building's installation for short-circuit (overcurrent) protection. Ensure that a fuse or circuit breaker no larger than 120 VAC, 15A U.S. (240 VAC, 10A international) is used on the phase conductors (all current-carrying conductors).

• French

Attention: Pour ce qui est de la protection contre les courts-circuits (surtension), ce produit dépend de l'installation électrique du local. Vérifier qu'un fusible ou qu'un disjoncteur de 120 V alt., 15 A U.S. maximum (240 V alt., 10 A international) est utilisé sur les conducteurs de phase (conducteurs de charge).

• German

Warnung: Dieses Produkt ist darauf angewiesen, daß im Gebäude ein Kurzschluß- bzw. Überstromschutz installiert ist. Stellen Sie sicher, daß eine Sicherung oder ein Unterbrecher von nicht mehr als 240 V Wechselstrom, 10 A (bzw. in den USA 120 V Wechselstrom, 15 A) an den Phasenleitern (allen stromführenden Leitern) verwendet wird